Marta Alexandra Simões Catalão
Bachelor's degree in Biology

Screening of Exopolysaccharides and Polyhydroxyalkanoates producing bacteria isolated from SPUR of Estremadura

Dissertation for Master's degree in Biotechnology

Supervisor: Doctor Cristiana Andreia Vieira Torres,
UCIBIO-REQUIMTE, FCT-UNL

Co-supervisor: Doctor Susana P. Gaudêncio,
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Jury
President: Professor Ana Cecília Afonso Roque
Examiner: Doctor Francisco Xavier Inês Nascimento
Vogal: Doctor Cristiana Andreia Vieira Torres

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LOMBADA

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Abstract

Marine environments represent a huge biodiversity due to their several different habitats, being abundant in unknowns' microorganisms and a source of new biomolecules, namely exopolysaccharides (EPS) and polyhydroxyalkanoates (PHA), with unique physical characteristics with application in pharmaceutical industry, medical, cosmetics, food and in bioplastic production. EPS are biopolymers excreted by bacteria when exposed to extreme conditions. In contrast, PHA are biodegradable bioplastics accumulated by bacteria as carbon stock and/or energy, in conditions with limited growth and carbon source in excess.

The objective of this work was evaluating the capacity of bacteria isolated from marine sediments collected off the Estremadura SPUR, Portugal, to produce biopolymers. These sediments were collected from deep-sea, in depths between 230-400 meters. In total, 67 strains were isolated and tested in 30 mL cultures with promising results, where all bacteria showed capacity to produce EPS and PHA. In consideration of growth and productivity, some bacteria were tested in 200mL. According to these results, bacteria that showed the most promising results in relation to growth (> 0.80 g/L), EPS production (> 0.28 g/L) and PHA accumulation (fluorescence when coloured with Nile Blue dye) were studied in biorreator. The pH was controlled (7), temperature (30ºC), aeration (1vvm) and pO_2 (20%) over the assay.

The best results were obtained by SPUR-41 with a production of 1.83 g/L of EPS and a specific growth rate of 0.25 h^{-1} when cultivated in medium containing 20 g/L of glucose, 4 g/L of yeast extract and 2 g/L of peptone in bioreactor. The composition of biopolymers and taxonomic characterization of bacteria were performed to improve the characterization of the full production process.

Keywords

Polyhydroxyalkanoates; Exopolysaccharides; Marine bacteria; Co-production; Screening

Resumo

Os ambientes marinhos apresentam uma grande biodiversidade devido aos seus diferentes habitats, sendo ricos em microrganismos desconhecidos e uma fonte de novas biomoléculas, nomeadamente biopolímeros, como exopolissacáridos (EPS) e polihidroxialcanoatos (PHA), com características físicas únicas para utilização na indústria farmacêutica, médica, cosmética, alimentar e na produção de bioplásticos. Os EPS são biopolímeros excretados pelas bactérias quando sujeitas a condições extremas. Por outro lado, os PHA são biopoliésteres biodegradáveis, acumulados pelas bactérias, como reservas de carbono e/ou energia, em condições de crescimento limitado e fonte de carbono em excesso.

Este trabalho teve como objetivo avaliar a capacidade de bactérias isoladas a partir de sedimentos marinhos recolhidos de sedimentos marinhos do SPUR da Estremadura, Portugal, produzirem biopolímeros. Estes sedimentos foram extraídos do fundo do mar, a profundidades entre 300 e 400 metros. No total, 67 estirpes foram isoladas e testadas em culturas de 30mL com resultados promissores, sendo que todas as bactérias demonstraram capacidade para produzir EPS e PHA. Tendo em conta o crescimento e a produtividade, algumas estirpes foram testadas em 200mL. Com base nestes resultados, as bactérias que demonstraram resultados mais promissores relativamente ao crescimento (> 0.80 g/L), produção de EPS (> 0.28 g/L) e produção de PHA (fluorescências quando coradas com Azul do Nilo) foram estudadas em biorreator, tendo sido controlado o pH (7), T (30ºC), arejamento (1vvm) e o pO_2 (20%) ao longo de todo o ensaio.

Os melhores resultados foram obtidos pela estirpe SPUR-41 que obteve uma produção de 1.83 g/L de EPS e uma taxa especifica de crescimento de 0.25 h^{-1} quando cultivada em meio contendo 20 g/L de glucose, 4 g/L de extrato de levedura e 2 g/L de peptona em biorreator. A composição dos biopolímeros assim como a caracterização taxonómica das bactérias foi realizada de modo a aumentar a caracterização do processo global de produção.

Palavras chave

Polihidroxialcanoatos; Exopolissacáridos; Bactérias marinhas; Coprodução; Screening

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List of Abbreviations

C/N – ratio carbon/nitrogen

CAGR – Compound annual growth rate

CDW – Cell Dry Weight

DO – Dissolved oxygen concentration

EPS – Exopolysaccharide

FDA – Food and Drug Administration

FSW – Fresh sea water

GC – Gas chromatography

HA – Hidroxyalkanoates

HB – Monomer of hydroxybutyrate

HPLC – Hight Performance Liquid Chromatography

HV - Monomer of hydroxyvalerate

mcl-PHA – Medium chain length polyhydroxyalkanoates

n.a. – Data not available

P(3HB) – Poly(3-hydroxybutyrate)

P(3HB-co-3HV) - Copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate)

P(3HHx) - Poly(3-hydroxyhexanoate)

P(3HHx-co-3HO) – Copolymer poly(3-hydroxyhexanoate-co-3-hydroxyoctanoate)

P(3HO) - Poly(3-hydroxyoctanoate)

P(3HV) – Poly(3-hydroxyvalerate)

PHA – polyhydroxyalkanoates

rpm – Rotation per minute

scl-PHA - Short chain length polyhydroxyalkanoates

SSW – Synthetic sea water

UVB - Ultraviolet B

Chapter 1

Introduction

1.1 Marine Environment

The hydrosphere marine environment covers approximately 70% of Earth's and represents 50% of the total biomass of prokaryotes in the world. It is composed of diverse and complex ecosystems that exhibit extremely variable conditions of pressure, temperature, pH, nutrients availability, chemical composition and salinity. Due to all of these parameters and the fact that housing the most diverse groups of flora and fauna, the oceans represent a vast and exhaustive source of natural products in the globe (Satpute et al., 2010).

Microorganisms populate every niche of Earth. The microorganisms like bacteria are the widest sources in the production of new biomolecules with potential for biotechnological applications in all sectors of industrial activity. So, it is expected that bacteria isolated from marine environments have developed biomolecules with diverse composition, distinctive and unique properties, which are not often in terrestrial organisms allowing their survival in adverse conditions of marine environment (Roca et al., 2016; Fenical, 1993). The world's oceans are still forgotten and little unexploited, remaining an open and very promising research field for the discovery of biomolecules with biotechnological applications for several industries like polysaccharides and polyhydroxyalkanoates (PHA) (Dufourcq et al., 2013).

1.2 Polysaccharides

The word biopolymer means polymers that have their origin in biological systems, instead of chemical polymers, using biological raw material such amino acids, sugars or natural fats (Ates et al., 2015). Biopolymers have many advantages over chemical polymers like ease of biodegradability, high efficiency, non-toxicity and non-secondary pollution. The most abundant group of natural polymers are the polysaccharides (More et al., 2014; Elnashar, 2011).

Polysaccharides are biodegradable polymers produced by many living beings and its possible to obtain them from plants (eg. starch and pectins), animals (e.g. chitin), algae (e.g. carrageenan, alginate, agar) or microorganisms (e.g. xanthan, gellan, pullulan) (Kumar et al., 2007). They are biopolymers with high molecular weight (10^4-10^7) and high structural diversity. The chemical structure of a polysaccharide comprises one or more monomers, linked to each other through glycosidic bonds, often forming repeating units and presenting different degrees of ramification (Reis et al., 2008). The monomers that compose polysaccharides are neutral sugars (e.g., galactose, glucose), acidic sugars (e.g., glucuronic acid, galacturonic acid) and amino sugars (e.g., N-acetyl-glucosamine, N-acetylgalactosamine). However, many polysaccharides possess non-sugar substituents called organic acyl groups (acetyl, succinyl, pyruvyl) and inorganic groups (sulphate, phosphate) (Sutherland, 1982). They can be constituted of one type of monosaccharides, in homopolysaccharides, or comprise two or more, in heteropolysaccharides (Casillo et al., 2018).

In nature, polysaccharides have different functions like structural element, maintaining mechanical form and rigidity of the living cells (e.g. cellulose and pectin in plant cell wall; chitin in

arthropod exoskeletons), as energy reserve substance (e.g. starch in plants; glycogen in animals and microorganisms) or adhesion and protective barriers against harmful conditions (microorganisms) (Kaplan,1998; Kumar et al., 2007; Elnashar, 2011).

Microorganisms are capable to produce three types of polysaccharides: intracellular, structural and extracellular polymers, the last ones are produced in response to environmental stresses and can be found attached to the cell wall or surrounding the organisms, called exopolysaccharides (EPS) (Vijayendra et al., 2013). The exopolysaccharides extracted from bacteria have improved physical and chemical properties when compared to the ones extracted from plants or algae (Torres et al., 2015).

1.2.1 Exopolysaccharides

Exopolysaccharides (EPS) are polymers with interesting and unique properties that are produced by a diverse group of microbial systems and secreted into the external environment as a slime that is weakly connected to the cell surface or synthesized extracellularly by cell wall-anchored enzymes (Nwodo et al., 2012; Torres et al., 2010).

Exopolysaccharides are high molecular weight carbohydrate polymers that have high chemical and structural diversity. They are produced by cells as a protective barrier against harmful conditions, surrounding the microbial cells, which are important for biofilm formation, constituting 40% to 90% of the total organic matter of a biofilm (Schmid and Sieber, 2015; Flemming et al., 2001). Although, still not completely elucidate the factors leading to EPS synthesis, several important physiological functions have been proposed: protection against environmental pressures (e.g. osmotic stress, temperature, pH, damage by Ultraviolet light, heavy metals, oxidants, desiccation, salinity), cell adherence to surfaces, carbon or water storage reserves and cell-to-cell interaction (Delattre et al., 2016; Donot et al., 2012; Papinutti, 2010; Rehm, 2010).

The most common sugar residues in EPS structures are glucose and galactose, but certain EPS like L-fucose, L-rhamnose or uronic acids, called rare sugars, are also present. Although it is not yet well understood why, they may provide an extra biological protection to the cells (Roca et al., 2015). This makes rare sugars attractive for a range of applications because they have many interesting properties. Other organic or inorganic substituents, such as phosphate, sulphate, succinic, acetic and pyruvic acids can also be found (Kenne et al., 1983).

1.2.1.1 Applications of EPS

In the last few years, the interest in polysaccharides has increased in various fields as they offer a great diversity of applications and due to their environmentally friendly properties. Their physical and chemical characteristics make possible the diverse functional properties like thickening, film forming, gelling, emulsion stabilizing, flocculating, nano/microstructures

production abilities such as anti-inflammatory substances and antioxidant (Kumar et al., 2007; Dumitriu, 2004).

EPSs are considered products with high value applications due to their properties like biocompatibility and biodegradability and to their distinct physical-chemical properties like water retention capacity and rheological (e.g. emulsifying, thickening and stabilizing capacity). They can be used as adhesives, absorbents, lubricants, soil conditioners, drug delivery vehicles, textiles, high-strength materials, emulsifiers, viscosifiers, suspending, and chelating agents (Ates, 2015). This physic-chemical property enables a wide range of industrial application for EPS in sectors like food, paper, paint, cosmetics, pharmaceutical and agriculture. As they are biocompatible these may be used in biomedical applications (e.g. scaffolds or matrices for tissues engineering, wound dressing and drug delivery) (Rehm, 2010). The high suspension stability of EPS provides their applications in pharmaceutical cream formulations, barium sulphate preparations, in cosmetic industry and in toothpaste formation (Tabibloghmany and Ehsandoost, 2014). They interact with the water molecules, control the rheological properties and improve product stability and quality.

In fact, EPS can also be used to treat environmental concerns like bioremediation of pollutants, detoxification of heavy metals, oil recovery and wastewater treatment. Mainly, EPS produced by microorganisms that live in extreme environments, such as marine environments with great affinity for heavy metals. They have the capacity to adsorb metal cations, among other dissolved substances, what can be useful in the treatment of wastewater systems, as biofilms are able to bind to and remove metals such as copper, lead, nickel, and cadmium (Pal et al., 2008).

Despite these potential properties, bacterial EPS currently represents a very small fraction of the global polymer market, mostly because their production costs.

1.2.1.2 Commercial Exopolysaccharides

A huge diversity of microbial EPS structures has been reported but only few, like xanthan gum, levan and dextran, are well known industrial polysaccharides with considerable markets (Freitas et al., 2017). The polysaccharide industry continues to be dominated by products obtained from algae and plants.

The first bacterial EPS to be commercialized was dextran, a homopolysaccharide, composed by monomers of alpha-D-glucose produced by lactic acid bacteria of the genera *Leuconostoc*, *Streptococcus*, *Weisella*, *Pediococcus* and *Lactobacillus*. Is characterized by a polymer with flexible structure, as result of free rotation of glycosidic bonds, water soluble and is used like a functional hydrocolloid. In addtion, dextran is Generally Regarded as Safe (GRAS) and commonly used in different food products manufacturing, cosmetic and medical applications and were approved by the FDA (Food and Drug Administration) (Patel and Prajapati, 2013). It is also used as an emulsifier, thickener and stabilizer.

Xanthan is a heteropolysaccharide commercially produced by the *Xanthomonas*

campestris (Fernandes et al., 2009). Is composed of monomers of glucose, mannose and glucuronic acid. It is widely used in the food, pharmaceutical industry and cosmetic due to its thickening, stabilizing and emulsifying properties (Ates., 2015). In 1969, xanthan was approved by the FDA for food use.

A homopolysaccharide widely used in the food, cosmetic and pharmaceutical industry is levan. Levan is composed of fructose monomers and produced by various bacteria such as *Halomonas smyrnensis, Bacillus subtilis and Zymomonas mobilis* (Öner et al., 2016). It has a high solubility in water and oils, has a great capacity for adhesion, biological activity, emulsifying capacity and film formation (Ates., 2015). It is also important to intensively work to discover new bacteria strains able to produce rare sugars, highly valuable but shortage, because of the growing interest of the industries due to their possible applications. Rare sugars are monosaccharides that rarely appear in nature, like L-fucose, L-rhamnose or uronic acids and its presence confer additional biological properties compared to those composed of more common sugar monomers (Torres et al., 2011). These sugars have many interesting physical and bioactive properties, making them attractive for various fields of applications, such as biomedicine, pharmaceuticals or cosmetics.

A more intensive study of EPS derived from bacteria is important because of their unique properties. Therefore, it is important to improve its production process, clarifying the function that they have in the organism and identifying and realizing the biosynthesis pathways involved in their synthesis (Freitas et al., 2017).

1.2.1.3 Biosynthesis of EPS

The mechanism of EPS biosynthesis depends on the type of polysaccharide that is being produced and is a complex process due to the involvement of several enzymes. The production of EPS demands a high energy cost due to the wide quantity of carbon that is consumed, however, gains in EPS production (e.g., increased bacterial growth and survival) turn out to be advantageous (Sutherland, 1982; Wolfaardt et al., 1999). EPS synthesis has 3 distinct phases: absorption of the carbon source, intracellular synthesis of the polysaccharide and its output from the cell (Vandamme et al., 2002).

Biosynthesis of EPS starts when carbon substrate is available as a precursor to the cell, which can enter in an active or passive way. After that, the substrate is catabolized by periplasmic oxidation or intracellular phosphorylation (Gupta et al., 2016). The periplasmic oxidative pathway exists only in certain bacteria, while the intracellular phosphorylate pathway is present in almost every bacterium but both of these systems can work concurrently, if there is enough substrate availability (Freitas et al., 2011). For the synthesis to occur it is necessary activated precursors, monosaccharides with high energy value must be produced (NDP-sugar).

EPS are hydrophilic (derived from hydroxyl and carboxyl groups), high molecular weight polymers, which are aggregated in the cytoplasm and have to cross the cell envelope without

damaging the properties of the bacteria barrier. For most Gram-negative bacteria, the pathway for biosynthesis and export of EPS can occur via one of two different pathways: the Wzx/Wzy-independent (ABC transporter-dependent) or the Wzx/Wzy-dependent pathway (Ates et al., 2015). In the case of Gram-positive bacteria, the synthesis occurs in the exterior of the cell and is mediated by a range of extracellular enzymes (Whitfield., 1988).

Marine bacteria, due to adverse conditions of aquatic ecosystems, produce a large diversity of extracellular polymers that constitute a substantial part of the dissolved organic carbon, in the form of EPS. Various studies have been carried out to elucidate the exact mechanisms of biosynthesis of EPS in bacteria, but mechanisms of biosynthesis of EPS in the marine bacteria have remained unclear.

1.2.1.4 EPS produced by marine bacteria

The research of marine bacteria is increasing due to the ability to survive in extreme conditions, as above-mentioned they can tolerate various conditions such as alkaline, hyper saline, fluctuation in temperature, pH, pressure and nutrient availability (Rampelotto, 2013). Extreme environments exert a pressure on bacteria for selection of new adaptive strategies leading to natural synthesis of novel bioactive compounds (Jensen et al., 1996; de Carvalho et al., 2010).

To date, there are several known bacteria isolated from extreme marine environments, such as thermophiles and halophytes, which are capable of producing EPS with interesting characteristics, although three main genera are commercialized (*Pseudoalteromonas* sp., *Alteromonas* sp. and *Vibrio* sp.) (Table 1.1) (Freitas et al., 2011). For example, a *Pseudoalteromonas* sp., was isolated by Roca et al., from ocean sediment from the Madeira Archipelago and is capable of producing an EPS that has rare sugars in its composition and have a production of 4.4g/L, the highest value so far obtained for a *Pseudoalteromonas* sp. strain (Roca et al., 2016).

One of the few marine bacteria EPS that is commercially available is Hyalurift®, a hyaluronic acid-like, produced by *Vibrio diabolicus* (Hall et al., 1999), a deep-sea bacterium. This showed a great potential in vitro regeneration of tissues, capable of restoring bone and skin, and the ability to accelerate in vitro collagen fibrillation and activate fibroblasts (Holmstrom, 1999). However, the first EPS derived from marine bacteria to be commercialized was the deepsane, produced by *Alteromonas macleodii.* Have high molecular weight and is composed of seven different types of monosaccharides: fucose, rhamnose, glucose, galactose, mannose, glucuronic acid and galacturonic acid (Le Costaouëc et al., 2012). This EPS is commercially available under the name of Abyssine ® and has wide importance to the cosmetic industry as it smooths and reduces irritation of sensitive skin against chemical, mechanical and UVB exposure (Lelchat et al., 2015).

Table 1.1 - EPS produced by marine bacteria

Bacteria	Source	EPS composition	Reference
Pseudoalteromonas sp. MD12-642	Marine sediments, Madeira Archipelago, Portugal	Galacturonic acid, glucuronic acid, rhamnose, glucosamine	Roca et al., 2016
Pseudomonas stutzeri 273	Marine sediments	Glucose, rhamnose, mannose and amino sugars	Sun et al., 2016
Alteromonas sp. JL2810	Seawater, South China	Rhamnose, mannose and glucuronic acid	Zhang et al., 2015
Vibrio harveyi strain VB23	Mandovi and Zuari estuaries, Goa, India	Galactose, glucose, rhamnose, fucose, mannose, ribose, arabinose, xylose	Bramhachari et al., 2016

1.2.1.5 The advantage of producing EPS from microorganisms compared to production in plants and animals

The common sources of industrial polysaccharides are plants, animals, fungi, algae and bacteria. In the last decades, research has focused on the capabilities of bacteria to secrete exopolysaccharides, because these polymers differ from the commercial ones derived essentially from plants or algae in their numerous valuable qualities or discovered new properties. However, the biopolymers market remains dominated by those produced by algae and plants, where microbial polysaccharides still represent a small fraction. The major reason for this to happen is the high cost of bacterial EPS production, limited by the elevated price of the commonly used carbon sources (e.g. glucose, fructose, sucrose), alone, the cost of substrate represents up to

40% of the total production costs (Roca et al., 2015). However, this limitation can be overcome by the use of low-cost carbon sources from agro and industrial wastes or by-products and optimize EPS production through downstream processes (Reis et al., 2008; Kumar et al., 2007). In another way, plants and animals production is affected by environmental factors, the bioprocesses are difficult to control and do not guarantee fast and reproducible production (Roca et al., 2015). In contrast, bacteria have the advantage to produce high structural reproducibility due to the severely monitored conditions of microorganism growth, usually have higher growth rates and are more prepared for enhancing growth and/or production by changing the cultivation conditions (Freitas et al., 2011). For these reasons, the research of new microorganisms for obtention of polysaccharides is nowadays in high demand (Casillo et al., 2018).

1.3 Polyhydroxyalkanoates

Besides exopolysaccharides bacteria are also able to produce other biopolymers like Polyhydroxyalkanoates (PHA), this polymer is biodegradable and mouldable when applying heat and pressure and is a potential alternative to conventional petroleum-based plastics.

Polyhydroxyalkanoates are linear polyesters of various hydroxyalkanoates (HA), connected by an ester bond (Figure 1.1). They are produced by some species of bacteria from renewable sources (sugars and fatty acids) with high potential applications and are accumulated in the cytoplasm as carbon and energy reserve material. The PHA is reserved in a form of insoluble granules when an essential nutrient is limited, such as oxygen, nitrogen or phosphorus, and in presence of excess carbon source (Lee, 1996). Some bacteria can accumulate as much as 90% of their cell dry weight in PHA (Mozejko-Ciesielska et al., 2016).

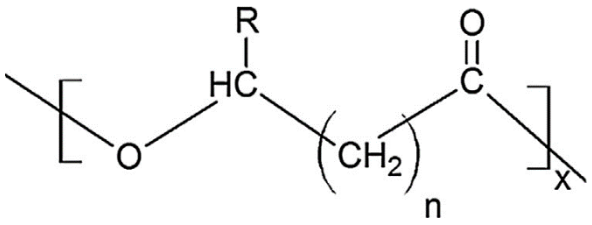

Figure 1.1 - Structure of polyhydroxyalkanoates: n-varies 1 to 4, x varies from 100 to 300000 (is the monomers number), R is the side chain of each monomer (adapted from Mozejko-Ciesielska et al., 2016).

PHA are mainly classified into two distinct groups, depending on their carbon numbers, scl-PHAs (short chain length PHA) consist of 3-5 carbon atoms. Examples of this class include poly(3-hydroxybutyrate), P(3HB) and poly(3-hydroxyvalerate), P(3HV). Another group is mcl-PHA (medium chain length PHAs) are composed of 6-14 carbon atoms. Examples include photopolymers poly (3-hydroxyhexanoate), P (3HHx), poly (3-hydroxyoctanoate), P (3HO) and heteropolymers such as P (3HHx-co-3HO). Due to the diversity of monomeric units, PHA have

different material properties, scl-PHA are rigid, brittle and crystalline thermoplastics but on the other hand, mcl-PHA are elastic and flexible, characterized by low crystallinity degrees and low melting temperatures (Laycock et al., 2014; Rai et al., 2011). The produced monomers depend on the bacterial strain, the carbon source and PHA synthases substrate specificity (Anjum et al., 2016).

The first PHA ever identified and the most widespread, studied and best characterized, was obtained by *Bacillus megaterium* and named poly-3-hydroxybutyric acid (P(3HB)). This homopolymer is highly crystalline (55–80%), rendering it brittle and giving it little strength to resist impact. These characteristics limit its uses. The glass transition temperature of P(3HB) is approximately 5ºC and its melting point is approximately 175ºC (Reis et al., 2003). However, the incorporation of 3-hydroxyvalerate (HV) units in PHB, results in the production of the copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate), or else P(3HB-co-3HV). P(3HB-co-3HV) is a material that becomes tougher, more flexible, and broader to thermal processing when its molar fraction in the copolymer increases (López et al., 2015). From all bioplastics, this is one with more promising properties for biomedical applications, due to its null toxicity and high biocompatibility with many diverse types of cells. Besides, there is a wide range of applications ranging from everyday disposable objects from bags, containers, packaging, cosmetics, hygiene products (towels, diapers, and handkerchiefs) to products that require high mechanical resistance such as helmets for cyclists, and printed wiring boards (for electronics), and various car panels (Rivera-Briso et al., 2018)

Exposure to soil, compost, or marine sediment can degrade PHA. Biodegradation depends on a multiplicity of factors including microbial activity of the environment, the amount of exposed surface area, temperature, pH, polymer composition, as well as crystallinity. Biodegradation of PHA under anaerobic conditions results in carbon dioxide and methane on the other hand, carbon dioxide and water are produced during aerobic conditions. According to studies, 85 % of PHA can be degraded in seven weeks (Farinha, 2009; Reddy et al., 2003).

More than 100 different monomers were identified as constituents of PHA in various bacteria. However, only a few of these have been produced in sufficient quantities (Zinn et al., 2001) to allow the characterization of their physical properties and to evolve potential applications.

1.3.1 PHA properties and applications

PHA have drawn great interest due their properties like biodegradability, water insoluble, biocompatibility, non-toxic and own thermic and mechanical properties similar to petrochemical plastics, namely polypropylene. Further, they are stable in air, inert, and can be completely degraded by the microorganisms of all environments (Anjum et al., 2016). Due to these properties, it is used in several industries, namely packaging (e.g., food containers, plastic bottles), biomedical (e.g., implants, drug delivery devices), fuel industry (production of biofuel), electronic

industry (e.g. mobile phones) and agriculture (e.g. encapsulation of seeds and fertilizers, long term dosage carriers of insecticides and herbicides) (Ali and Jamil, 2016; Cruz et al., 2016; Takahashi et al, 2017; Chen, 2009; Verlinden et al., 2007).

PHA have attracted much attention in recent years because of their similarities with conventional petroleum-based plastics properties and fully independent petroleum sources, so the majority of possible applications for PHA are the replacement of the petrochemical polymers market in the near future (Thomas et al., 2020).

According to “Report European Bioplastics, 2019”, PHA are considered innovative biopolymers, with a growth in the field of bio-based and biodegradable plastics market. In 2019, PHA represented 1.2 % of the global 2.11 million tonnes of bioplastics produced. It is estimated that the production capacities can triplicate in the next five years. Inclusive, a recent report, publish in 2019, predicts that the global PHA market is expected to reach US$98 million by 2024, from an estimated US$57 million within 2019, characterized by a compound annual growth rate (CAGR) of 11.2%. (Polyhydroxyalkanoate (PHA) Market).

The only disadvantage, which prevents petrochemical polymers from being replaced by PHA, is the fact that production costs are very high. A solution to this problem is the use of low-cost substrates, genetic engineering, bioinformatics and/ or isolate new bacteria that are able to use cheap substrates and produce PHA with good productivities and interesting properties (Wecker et al., 2015; Thomas et al., 2020). Is important to invest in PHA-producing bacteria because they have been reported to be found in various environments such as wastewater, industrial waste, municipal waste, soil, compost, hot spring water, freshwater and marine water (Urairong H., & Rodtong, S., 2012).

1.3.2 Marine microorganisms that produce PHA

Marine environment, as already mentioned, is composed of diverse and complex ecosystems that exhibit extremely variable conditions, what makes bacteria existent in these media potential sources of new biopolymers, including PHA (López-Cortés et al., 2008). Although, the marine environment has been little explored in terms of prospecting for PHA producing organisms, several authors described halophilic strains isolated from various marine ecosystems (e.g. marine mat, sea water) as PHA producing bacteria (e.g. *Vibrio nereis*, *Vibrio natriegens*, *Halomonas boliviensis*, *Halomonas campaniensis* LS21, *Halomonas hydrothermalis* SM-P-3M, *Haloferax mediterranei*) (Chien et al., 2007; Kavitha et al., 2018; Takahashi et al., 2017; Thomas et al., 2020) (Tabela 2.1). So far, *Haloferax mediterranei* is one of the best studied PHA producers, some studies have shown their ability to accumulate high PHA contents utilizing low-cost feedstocks like vinasse, stillage and cheese whey (Table 1.2) (Bhattacharyya et al., 2012; Bhattacharyya et al., 2014; Chien et al., 2007; Pais et al., 2015; Shrivas et al., 2010).

Table 1.2 - Characteristic parameters describing PHA production from different types of marine bacteria.

Strain	Marine Source	Carbon Source	PHA	CDW (gL^{-1})	PHA (gL^{-1})	PHA (%)	$Y_{P/S}$	Reference
***H. mediterranei* DSM 1411**	n.a	25% pre-treated vinasse	PHBV (86-14)	n.a	19.7	70	0.87	Bhattacharyya et al., 2012
	n.a	Rice-based ethanol stillage	PHBV (85-15)	n.a	16.4	71	0.35	Bhattacharyya et al., 2014
	n.a	Hydrolyzed cheese whey	PHBV (98.5-1.5)	7.5	n.a	54	0.78	Pais et al., 2015
***Halomonas hydrothermalis* SM-P-3M**	Sea water	Biodiesel byproduct containing crude glycerol	PHB	n.a	n.a	75.80	n.a	Shrivastav et al., 2010
***Vibrio* sp.**	Marine sediment	Sucrose	PHB	n.a	n.a	45.50	0.37	Chien et al., 2007

n.a – Data not available

The information described in table 1.2 shows that PHA produced by marine bacteria from extreme environments have great potential to be used and commercialized in biotechnological applications. The use of halophilic microorganisms that survive in environments with high concentrations of salts minimizes the risk of microbial contamination in the culture medium and, in some cases, sterilization is not necessary. Another advantage is minimal freshwater consumption, due to its substitution with seawater for medium preparation. In addition, downstream processing costs can be reduced by treating the bacteria cells with salt-deficient water in order to cause hypo-osmotic shock (Kourmentza et al., 2017; Quillaguamán et al., 2010).

1.4 Estremadura SPUR

The bacteria used in this study were isolated from Estremadura SPUR sediments. Estremadura SPUR is a promontory located on the continental shelf, off the coast of Portugal, between Cabo da Roca and Cabo Carvoeiro with an area of 3583 Km^2, oriented in the east-west direction. This promontory separates the Iberian abyssal plain to the north from the Tagus abyssal plain to the south (Duarte, 2015).

The Estremadura SPUR area is mainly composed of two distinct areas: internal sector, located about 120-130 meters below sea level and where the coastal plateaus Pêro da Covilhã and the mountain of Camões are located, being separated by the basin of Ericeira Sea and the external sector, located up to about 500 meters below sea level, where the shallow immersion

surface, the Monoclinal of Lourinhã, is positioned (Duarte, 2015; Balsinha et al., 2014). Due to the geomorphological diversity in the places that constitute it (fine, coarse sand and muddy areas of terrestrial origin but also biogenic and autogenic in the deepest places), there is a great diversity of sediments in the Estremadura SPUR.

Marine sediments are rich in organic matter that vary in depth and from place to place. Sediments represent in geological phenomena a diversity of bacterial flora (Fenical, 1993). This diversity can favour the growth of microorganisms that produce value-added biopolymers such as EPS and PHA.

1.5 Objective

Marine habitats (deep sea, hydrothermal vents, cold seeps, volcanic and hydrothermal marine areas, salt lakes, marine sediments) are an important source of new bacteria that need to support extreme conditions and create defence, like the production of natural polymers, to survive. To date, various marine bacteria are known for production of biopolymers like polysaccharides with greatly variable composition, structure, and functional properties. As they are produced in extreme conditions, these biopolymers can have new properties to be used in a wide range of biotechnological applications in all sectors of human activity. The polysaccharides are biopolymers non-toxic and biodegradable, a quality that turns them into an attractive biomaterial for sustainable development.

In recent years, sustainability, environmental concerns and green chemistry have played an important role in guiding the development of the next generation of environment-friendly materials, products and processes. The increase of products derived from petrochemicals, non-degradable wastes is an emerging concern, which leads to attempts to find alternatives to replace it, such as bioplastic, like PHA, which, as described above, have similar properties to petroleum-based products but unlike them, are biodegradable.

In this study, 67 bacteria were isolated from sediments obtained from various depths in SPUR from Estremadura, located in Atlantic Ocean, 170Km off the Portuguese coast. These samples were collected in 14 stations in seabed at depth between 230 and 400 meters to obtain a larger diversity of new microorganisms and possibly new strains that produce a higher quantity of EPS or new structures of PHAs.

The objective of this study was to screen marine-derived bacteria strains for the capability of producing innovative biopolymers like EPS and PHA. This work focused on upstream steps, firstly a screening in 30 mL cultivation medium. The effect of different media composition was evaluated as well as the amount and composition of EPS and PHA. Bioreactors for the best biopolymer producers and the taxonomic characterization of the selected strains were performed.

Duo to time limitation in laboratory, consequence of COVID-19 pandemic, work was affected and influenced some data collection.

Chapter 2
Materials and methods

2.1 Collection and preservation of microorganisms

2.1.1 Sample collection

The bacteria used for this study were isolated from marine sediments collected in the Atlantic Ocean, off Portugal Coast, specifically from the Estremadura SPUR using a claw Smith McIntyre Grab (200-400 m depth). The sediment samples were provided by Dra. Clara Rodrigues by CESAM from Universidade de Aveiro (project PTDC/ GEO-FIQ/5162/2014).

2.1.2 Preservation

In this study, 67 strains of marine bacteria were screened for the production of exopolysaccharides (EPS) and polyhydroxyalkanoates (PHA). The strains were preserved in cryovials with 20% (v/v) glycerol and stored at -80ºC.

2.2 Screening of marine bacteria to produce EPS and PHA

2.2.1 Cultivation medium

Media A1 in synthetic sea water (Table 2.1) was adapted from Roca et al., 2016 and used for the screening experiments. The composition of synthetic sea water (SSW) used was adapted from Akhlaghi et al. (2014) and the mineral components that comprise it are described in Table 2.1. All reagents constituents of medium A1 were dissolved in distilled water and the different solutions (1-5) present in Table 1 were autoclaved separately to avoid salts precipitation. The initial pH value was 7.

Agar plates were prepared using the same medium by adding 18.00 g/L of agar.

Table 2.1 - A1 medium (solid and liquid) of SSW solution

Chemical composition of A1 medium	**g/L**
Glucose (99%, Fluka)	10.00
Yeast Extract (99%, Panreac)	4.00
Peptone (99%, HIMEDIA)	2.00
Chemical composition of SSW	
Solution 2	
NaCl (99%, Panreac)	23.38
Na_2SO_4 (99%, Panreac)	3.41
$NaHCO_3$ (99%, Panreac)	0.17
Solution 3	
KCl (99%, Panreac)	0.75
Solution 4	
$MgCl_2$ (99%, LBCHEM)	4.24
Solution 5	
$CaCl_2$ (99%, Panreac)	1.44

To provide sterility and total dissolution, the media were autoclaved at 121ºC for 20 minutes (Uniclave 88, Portugal).

Culture reactivation was performed by growing the stock cultures in an agar plate for 24 hours at 30 °C, to obtain isolated colonies.

Every step involved in the handling of the bacterial strain was carried out in a laminar flow chamber (Heraeus SB 48, Germany).

2.2.2 Screening in 30 mL liquid culture medium

After obtaining isolated colonies, inoculum was prepared by placing an isolated colony from the plate into 30 mL of medium A1 in SSW. Incubation occurred for 24 hours in an orbital shaker (New Brunswick Scientific), at 30 °C and 200 rpm. Afterwards, 3 mL of the broth was transferred to a new falcon tube with 30 mL of equal medium.

The assays were performed for 48 hours and samples were taken periodically to assess cellular growth by measuring the optical density (600 nm), pH and Nile Blue staining.

2.2.3 Evaluation of cellular growth and biopolymers production in 200 mL-scale

Inoculum were prepared by growing an isolated colony in 50 mL of medium A1 for 24h, at 30ºc and 200 rpm in 100 mL shake flask. After 20 mL were transferred to 500 mL shake flask containing 200 mL of A1 medium. Samples were taken periodically to measure optical density at 600 nm, pH and Nile Blue staining. The runs were finished when the cellular growth ended.

2.2.4. Impact of using different growth media

Different media were evaluated for bacterial cultivation, namely, Media A1 prepared with deionized water.

Medium A1 (Roca et al., 2016) with fresh sea water (FSW) was composed by glucose (10 g/L), yeast extract (4 g/L) and peptone (2 g/L) and containing 750mL of filtrated sea water and 250mL of deionized water. The initial pH value was 7.

The experiments were conducted in 500 mL baffled shake flask containing 200 mL of the appropriate medium. An inoculum of 50 mL was used in all assays and the cultures were incubated in an orbital shaker at 200 rpm at 30 ˚C, for 24h. Samples were collected for determination of the optical density at 600 nm, Nile Blue staining, cell dry weight (CDW), PHA composition and EPS quantification.

2.3 Bioreactor cultivation

2.3.1 Medium preparation

The medium used for inoculum preparation in the batch reactor was A1, composed by: 20 g/L of glucose, 4 g/L of yeast extract, 2 g/L of peptone (solution 1 in table 1.1). These compounds were dissolved in FSW and deionized water in the proportion 75:25 (v/v). The medium was autoclaved at 121ºC for 30 minutes.

2.3.2 Inoculum preparation

One isolated colony from the selected strains was used to do the pre-inoculate in 50mL of medium in a 100mL shake flask. The pre-inoculum was incubated 24 hours at 30ºC and 200 rpm. After, 20mL from pre-inoculum were transferred to a 500mL shake flask with 200mL of A1 medium and were incubated 24 hours at 30ºC and 200rpm.

2.3.3 Operating conditions

This assay was performed in a 3L reactor (Solaris Biotechnology Jupiter Srl), with 2L of working volume where sterile conditions were maintained. The reactor was operated at 30ºC, pH was maintained at 7.0±0.10 by automatic addition of 2M HCl and 2M NaOH. The dissolved oxygen inside the bioreactor was maintained above 20% by automatically increasing the agitation from 200 rpm to 800 rpm. Air flow rate was maintained at 1vvm. Foam formation was suppressed by addition of an antifoam solution (BDH Prolabo – VWR). 200mL of inoculum in the exponential phase were used to inoculate the bioreactor, 20mL of samples were taken periodically during the time of bioreactor. The OD at 600nm was monitored immediately after sampling. Pellets were frozen for further analysis of biomass production and cell-free supernatant to sugars quantification, glucose and nitrogen concentration.

2.4 Analytical techniques

At the end of each run, culture broth samples (6 mL), were collected and centrifuged (Sigma 4 – 16 KS, Germany) for 15 minutes at 8000 rpm. Cell-free supernatant was used for EPS quantification. The cell pellets obtained, were washed once by resuspension in deionized water (6 mL) and centrifuged again (15 minutes, 8000rpm) and used for CDW and PHA quantification.

2.4.1 Cellular growth

Cellular growth was monitored during the experiment by measuring the optical density of the cultivation broth at 600 nm (OD600nm). All measurements were performed in duplicate. Samples were diluted with deionized water whenever necessary.

Biomass Quantification

The CDW was determined by gravimetry. The washed pellets were lyophilized (ScanVac CoolSafeTM, LaboGene), at -100 ˚C for 48 h. The CDW was obtained by weighing the lyophilized cell pellets. The analysis was performed in triplicate.

Nile Blue Staining

In an *Eppendorf* tube, 0.5 µL of Nile Blue was added to 0.5 mL cultivation broth sample, covered with aluminium foil, and placed in an oven at 100 ºC for 5 minutes. After this time, slides were prepared which were observed under the microscope (Olympus BX51 epifluorescence) under contrast light and fluorescent light, with a magnification of 100x.

Calculus

The specific cell growth rate (µ, h^{-1}) was determined by the following equation:

$$\mu = LN\frac{X_t}{X_0} \qquad \text{(Equation 1)}$$

When LN is natural logarithm, X_t is active biomass concentration in g/L at a certain time (t) in hours and X_0 is active biomass concentration (g/L) at the beginning of concentration.

The volumetric production of EPS (rEPS, g/L d^{-1}) were determined by the following equations:

$$r_{p\,EPS} = \frac{dEPS}{dt} \qquad \text{(Equation 2)}$$

where EPS is the EPS concentration (g/L) produced at time t (days)

The yields of active biomass on substrate ($Y_{x/s}$, g/g) and EPS on substrate ($Y_{\mathrm{eps}/s}$, g/g) were determined by the following equations:

$$Y_{x/s} = \frac{xf-xi}{sf-si} \qquad \text{(Equation 3)}$$

$$Y_{EPS/s} = \frac{EPSf-EPSi}{sf-si} \qquad \text{(Equation 4)}$$

where x_f and x_i are the final and initial active biomass concentration, s_f and s_i are the final and initial substrate, EPS_f and EPS_i are the final and initial EPS produced

2.4.2 Extraction and purification of EPS

The cell-free supernatants, from the different runs, were dialysed using 12-14 kDa molecular weight exclusion membranes (ZelluTrans Carl Roth - Regenerated Cellulose Tubular Membrane, Germany) against deionized water at room temperature under constant agitation, for 72 hours. Sodium azide (10ppm) was used to avoid biological degradation of the sample. The efficiency of dialysis was controlled by measuring the conductivity (FiveEasy F20, Brasil), when the value was below 10 µS/m, the dialysis was stopped and the purified polymer was freeze dried 48 hours (Scanvac, CoolSafe, Denmark). Then, the purified samples were weighed to determine EPS production and kept for further characterization.

2.4.2.1 Determination and quantification of sugar

The EPS was characterized in terms of its sugar monomers composition, according to Freitas et al., 2011. EPS dried samples (approximate 2-3mg) were dissolved in deionized water (5mL) and hydrolysed with trifluoroacetic acid (TFA) (0.1mL, 99%) in a dry bath at 120ºC for 2 hours. After cooling down to room temperature, 1 mL of the hydrolysed solution of each sample was filtered using Eppendorf membrane filter, centrifuged, and finally placed on the specific vial for the analysis.

Samples sugar monomers composition was determined by HPLC using a CarboPac PA10 250x4 column (Dionex) coupled with an AminoTrap 50x4 column (Dionex). The analysis was performed at 25 ºC with sodium hydroxide (NaOH, 18 mM) as eluent, at a flow rate of 1 mL/min. D-(+)-fucose (98%, Scharlau), D-(+)-glucose (99%, Fluka), D-(+)-galactose (99%, Fluka), D-(+)-mannose (99% Fluka), D-(+)-galacturonic acid (97%, Fluka), L-rhamnose monohydrate (99%, Fluka), D-(+)-mannose (99% Fluka), D-arabinose (99%, Sigma) and D-glucuronic acid (98%, Alfa Aesan), were used as standards in a range between 0.5 g/L a 0.005 g/L.

2.4.2.2 Determination and quantification of acyl groups

The EPS was characterized in terms of its sugar monomers composition, according to Freitas et al., 2011. EPS dried samples (approximate 2-3mg) were dissolved in deionized water (5mL) and hydrolysed with trifluoroacetic acid (TFA) (0.1mL, 99%) in a dry bath at 120ºC for 2 hours.

The hydrolysate was used for the identification and quantification of the acyl groups substituents. The analysis was performed by HPLC, with an IonPac ICE-AS1 9x250 mm column (Dionex), coupled to a Photodiode Array PDA ICS series (Dionex), using sulphuric acid (H_2SO_4 0.01 N) as eluent, at 30ºC, with a flow rate of 0.6 mL min^{-1}. The detection was performed at 210 nm. Pyruvate (Alfa Aesar, 98 %), succinate (Merck, 99.5%) and acetate (Sigma-Aldrich, 99.8%) solutions were used as standards in concentrations ranging from 1 to 100 ppm.

2.4.3 PHA quantification

To determine the polymer's content in the biomass and its monomer composition, gas chromatography (GC) analysis was performed, according to Cruz et al., 2016. Dried cell samples (1 to 10 mg) were hydrolysed in 2 mL of methanol acidic (20% (v/v) sulphuric acid (SIGMA-ALDRICH) in methanol (Fisher Chemical) and 2 mL of benzoic acid in chloroform (1 g/L) (SIGMA-ALDRICH), on an oil bath at 100˚C, for 4 h. Then, 1 mL of water was added. After separation of the organic and aqueous phases, the organic phase, with the resulting methyl esters, was transferred to vials and analysed by GC (430-GC, Bruker) with a Restek column of 60m, 0.53 mmID, 1 µM df, Crossbond, Stabilwax. The injection volume was 2.0 µL, with a running time of 32 min, a constant pressure of 14.50 psi and helium as carrier gas. The heating ramp was 0 to 3 min, a rate of 20˚C/min, until 100˚C, 3 to 21 min a rate of 3˚C/min until 155˚C and 21 to 32 min a rate of 20˚C/min until 220˚C. Hydroxybutyrate (HB) and hydroxyvalerate (HV) concentrations were determined through the use of two calibration curves, one for HB and other for HV, using standards (0.1-10 g/L) of a commercial co-polymer P(HB-HV) (88 %/12 %) (Sigma), and corrected using heptadecane as internal standard (concentration of approximately 1 g/L). Standards were processed in the same way as the samples. Mcl-PHA (VersaMerTM PHAs, PolyFerm Canada) standards were prepared at 1 g/L and then diluted to give concentrations in the range 0.05 and 1 g/L.

2.4.4 Glucose quantification

Glucose concentration in the cell-free supernatant was determined by high performance liquid chromatography (HPLC) with a VARIAN Metacarb 87H column coupled to an infrared (IR) detector, according to Torres et al., 2011. The analysis was performed at 50 °C using H_2SO_4, 0.01N, as eluent with a flow rate of 0.6 mL/min. Samples were prepared by diluting the cell-free supernatant, in the eluent (H_2SO_4, 0.01N), in a 1:20 (first sample of screening growth) and 1:10 (last sample of the screening growth) proportions. All samples were filtered using Eppendorf membrane filter (0.2 µm). A standard calibration curve was constructed using glucose (99%, Fluka), in a concentration range between 0.01 to 1g/L.

2.4.5 Total nitrogen

For total nitrogen determination, a kit (LCK 388, LATON®) with a detection range of 20-100 mg/L was used. The test solution (0.2 mL) was placed into a digestion flask; then, the reagents were added as described in the kit and the flasks were placed on the HT 200S (HACH®-LANGE) digester for 15 min at 100 °C. The flasks were cooled to room temperature. After cooling, the flasks were agitated, and 0.5 mL of the solution was transferred to a new flask and after 15 min the absorbance was read on a DR 2800 tm spectrophotometer (HACH®).

2.4 DNA extraction, 16S rRNA gene amplification, sequencing and taxonomic identification of bacteria

The taxonomic characterization of the selected bacteria was performed by Professor Rita Sobral at the MolMicro of Bacterial Pathogens Lab.

All strains were inoculated in 4 ml of medium A1 and incubated at 25ºC for 3 to 7 days with agitation (200 rpm). Total DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) as described by the manufacturer with some adjustments (Pereira et al., 2020). The 16S rRNA gene was amplified using primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGCTACCTTGTTACGACTT-3′) (Gontang et al., 2007) and NZYTaqII polymerase (NZYtech, Lisbon, Portugal). The PCR products were purified using the NZYGelpure clean-up kit following the manufactures protocols (Nzytech, Lisbon, PT). The purified products were quantified and sequenced by the Sanger method using the primers described above at STAB VIDA, Lda (https://www.stabvida.com). The 16S rRNA sequencing chromatograms were reviewed and consensus sequences for each forward/reverse pair were created with SeqManPro (Lasergene, DNAstar). All the sequences obtained were compared to the GenBank database by the BLASTN algorithm.

Chapter 3

Results and discussion

3.1 Screening in 30mL liquid cultivation with A1 medium

The 67 isolated strains were evaluated to assess the cellular growth and the ability of bacteria to produce biopolymers with added value, namely EPS and PHA. Therefore, replicates of all the strains were grown in 30 mL liquid medium A1 with synthetic sea water (SSW).

All the assays had a duration of 48 hours, after this time, bacteria stopped growth and remain in stationary phase, this could be related with a lack in some nutrient source. Thus, the choice of this cultivation time is to prevent bacteria from consuming the accumulated PHA.

Figure 3.1 shows that all bacteria were able to grow in medium A1. All the bacteria presented an exponential cellular growth at 24 hours of assay, after 48 hours the cellular growth had stopped for all of them and all the experiments ended. The values of cell dry weight (CDW) achieved by bacteria are within 0.26±0.15 g/L and 3.79±0.37 g/L, corresponding to bacterium SPUR-52 and SPUR-42, respectively. In fact, bacterium SPUR-42 presented the highest cellular growth, being the only with a CDW above 2 g/L. Most of the bacteria tested (~64%) attained CDW between 1 and 2 g/L. These are interesting results, since tests were performed without controlling essential parameters for bacteria's growth, such as pH and aeration. During the runs the culture broth pH reached values below 4. On the other hand, the low concentration of oxygen reaching the culture medium may also contribute for a low cellular growth. Agitation, an important mechanical factor for the growth of bacteria, may also had been a determining factor, since falcon tubes could not be placed in a way that all of them would be exposed to the same agitation conditions.

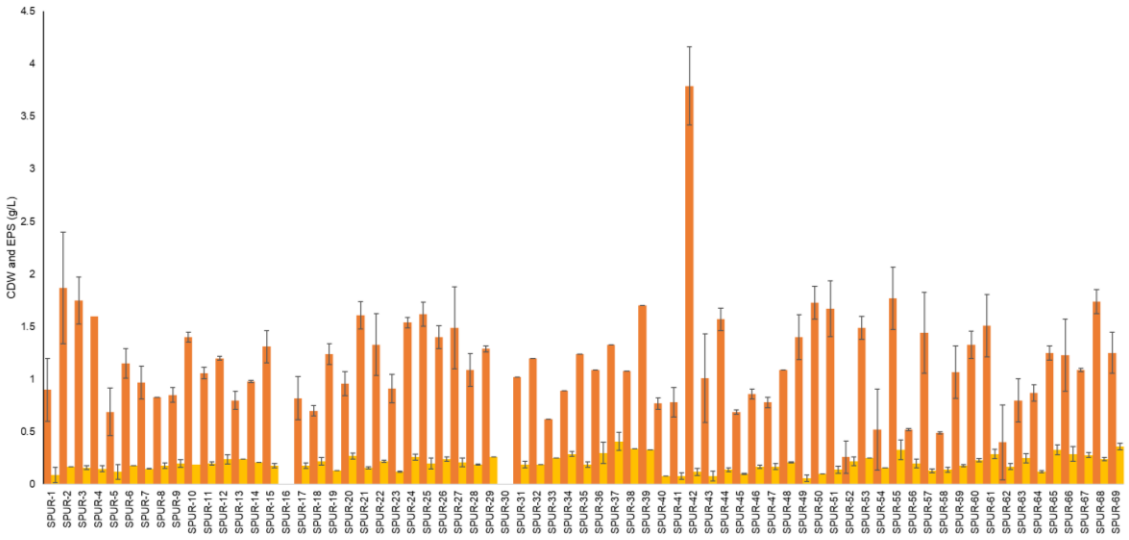

Figure 3.1 – Evaluation of CDW (●) and EPS (●) (g/L) produced by SPUR bacteria tested in 30mL of medium A1

Behind bacterial cellular growth, the biopolymer (EPS and PHA) production was also evaluated. Concerning EPS, all the bacteria were able to synthesize EPS, however in concentrations below 0.5 g/L. The bacteria SPUR-36, -37, -38, -39 and 69 synthesized the highest EPS concentrations (0.30 g/L-0.41 g/L). On the other hand, there were strains that showed very low production, with values below 0.10 g/L, such as SPUR-40, 43 and 49.

The low EPS concentrations attained may have been a consequence of the lack of control of the physicochemical parameters essential for their production. Indeed, the composition and the amount of EPS produced by microorganisms are directly influenced by medium components and fermentation conditions (pH, oxygen concentration, agitation, presence of carbon sources in sufficient quantities) (Nwodo et al., 2012). For EPS production, the optimum production pH is neutral (6.5-7.0). Since there was no control of this type, the pH measurements made over time showed that it reached very acidic values in some growth stages. Furthermore, many of the exopolysaccharide-producers require a constant pH for maximum production of exopolysaccharides (Morin, 1998).

Regarding PHA production, it was evaluated by optical microscope observations, using fresh culture broth samples stained with Nile Blue dye. Nile Blue is a lipophilic dye used to stain colonies and to distinguish between PHA-accumulating and non-accumulating strains (Spiekermann et al., 1999). This technique allows visualization of PHA accumulation under the light microscope (Ostle and Holt, 1982) through fluorescence emission. All SPUR-bacteria showed fluorescence when observed under an optical microscope after stained with the Nile blue dye. In figure 3.2, it is possible to observe the PHA granules accumulated inside the bacterial cells of the bacteria SPUR-8 and SPUR-24. It was not possible to quantify the PHA accumulated by the cells through gas chromatography due to the low PHA content and/or due to the low biomass concentration.

Figure 3.2 - Fresh samples of bacteria SPUR-24 (A) and SPUR-8 (B) stained with Nile Blue solution after 24 hours of incubation (100x).

3.1.1. EPS sugar monomer composition

In order to evaluate the composition of EPS produced by the different SPUR bacterial strains, previous purified (dialysis), samples were analysed by HPLC. It was found that the samples showed a wide variety of sugars, including neutral and acidic sugars (Figure 3.3). Most of the EPS synthesized are heteropolysaccharides containing 3 or more different monosaccharides, which is common is marine bacteria (Decho, 1990). The most predominant sugars were glucose and mannose. Inclusive, there are some bacteria whose composition in EPS is 100 %mol of glucose (monosaccharides of glucose), namely EPS SPUR-26, -40, -43, -44, -49, -57, -61 and -68. It was also observed that mannose is a dominant sugar, although it is not produced by all bacteria. However, these two are considered common sugars and are frequently found in EPS produced by other bacteria (Caruso et all.,2019; Bramhachari et al., 2006; Asker et al., 2018). Although there is a greater share of glucose and mannose, other sugars also exist, such as arabinose, rhamnose, fucose, glucuronic acid and galacturonic. The latter are considered rare sugars and have the potential to reveal unique properties to polysaccharides. It is noteworthy that glucuronic acids are in 69% of the samples and in some of them in quite remarkable concentrations (> 15 %mol), which would be expected since according to literature its synthesis by marine bacteria is common (Roca et al., 2016; Nichols et al., 2004). For example, EPS SPUR-62 has the highest amount of galacturonic acid, 27.82 %mol, while EPS SPUR-67 has the highest amount of glucuronic acid, 8.80 %mol. Another polysaccharide that has increased market value is fucose, which is a rare sugar difficult to obtain and occurred in EPS synthesised by some of the bacteria (Figure 3.3) but in higher concentrations in EPS SPUR-3, -41, -63 and -69 (between 2.8 %mol - 5.9 %mol).

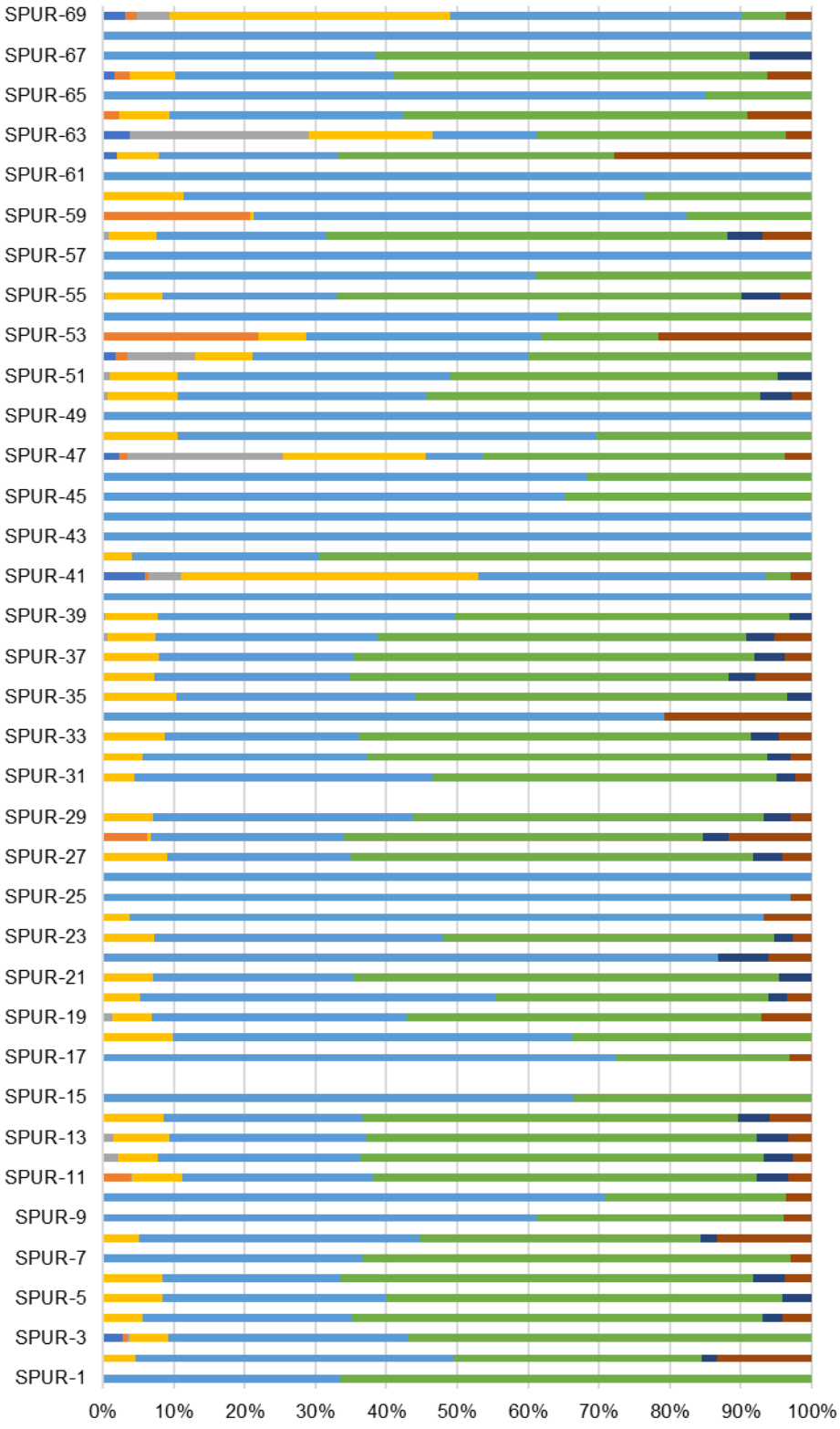

Figure 3.3 - Sugar monomer composition of EPS produced by SPUR-bacteria in 30mL of A1 medium (●) Fucose; (●) Rhamnose; (●) Arabinose; (●) Galactose; (●) Glucose; (●) Mannose; (●) Glucuronic Acid; (●) Galacturonic acid

3.2. 200 mL-scale up for growth and product determination

In order to improve cellular growth and production of EPS and PHA, the bacterial strains that attained the higher CDWs and the EPS production, were grown in 200 mL of medium A1. Furthermore, previous results (data not shown) and the slimy aspect of the colonies in the agar petri dish were also taken into consideration for the bacterial selection. From a total of 67 bacteria screened for EPS production and PHA accumulation, 25 were considered promising biopolymers producers.

3.2.1 Assays in medium A1 with SSW

Selected bacteria were grown in 200 mL of medium A1 using synthetic sea water (SSW) at 30ºC.

As in previous experiments, assays were performed during 48 hours. In the tests carried out in 30 mL of medium A1, all bacteria were able to grow. Actually, on average the selected bacteria attained higher cellular growth (1.42 g/L) than when grown in 30 mL (1.17 g/L) (Figure 3.4). The end of cellular growth occurred 24 hours after inoculation. These experiments had higher growth rates (0.05 – 0.12 h^{-1}) than the assays carry out in 30 mL (0.04 – 0-09 h^{-1}). The lowest growth was obtained by SPUR-1 and SPUR-28 with a CDW of 0.14±0.01 and 0.16±0.12 g/L, respectively. On the other hand, the highest CDW was obtained by bacteria SPUR-11, with a final biomass of 2.70 g/L. The general increase in cellular growth may have been a direct consequence of some essential parameters improvement, namely stirring and oxygen availability (enabled by the higher head space).

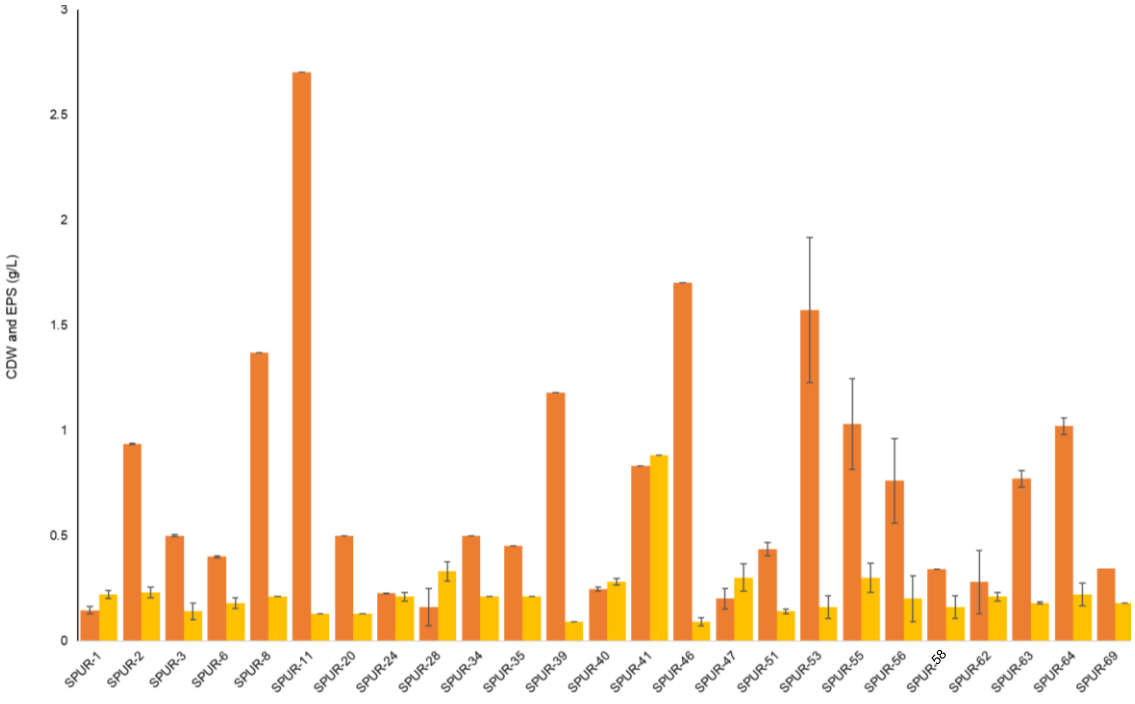

Figure 3.4. – Evaluation of CDW (●) and EPS (●) (g/L) produced by SPUR bacteria in 200 mL of medium A1.

In what concerns EPS production, the results obtained with the scale increase, did not result in a bigger EPS production. An example is SPUR-11 that showed a production of 0.20±0.01 g/L in 30mL but in larger scale showed a slight lower production of 0.13 g/L. However, the cellular growth attained was much higher (2.70 g/L), which suggest a metabolic preference to grow by using the nitrogen and carbon source available, or to accumulate PHA instead of secrete EPS.

As for cellular growth, in general, SPUR-bacteria exhibited a higher EPS production than in the experiments with 30 mL medium A1. Strain like SPUR-41, -47 and -55 reported the highest synthesis of EPS (0.88, 0.3±0.07 and 0.30±0.07 g/L, respectively). It is worth noting, that for SPUR-28, -41 and - 47, the EPS synthesis was greater than cellular growth. Even as for cellular growth the improvement in parameters such as stirring and oxygen availability may have contribute to the improvement in EPS synthesis. Although pH was not being controlled, during the runs the pH value practically did not change (pH 6-7), which may have impacted positively for a greater EPS production. Since according to literature, numerous microorganisms produce exopolysaccharide in media buffered at neutral pH. (Lee et al., 2001).

3.2.1.1. EPS sugar monomers composition

Figure 3.5 shows the sugar monomer composition of the EPS synthesized by the bacteria grown in 200 mL medium A1. It is possible to verify that the monomer composition have some changes when compared with EPS synthesized in the previous experiments.

The EPS SPUR-40, the only EPS with a composition of 100 %mol glucose in the smaller scale experiments, exhibited now a heteropolysaccharide EPS composed of 4.70 %mol fucose, 9.86 %mol galactose, 26.81 %mol glucose, 52.10 %mol mannose, 4.20 %mol glucuronic acid and 2.38 %mol galacturonic acid. The different composition of EPS can be explained because nutritional and culture conditions can affect the yield and quality of microbial exopolysaccharides, as well as the prediction of molecular mass, number of monosaccharide units and degree of branching of EPS (Kumar et al., 2007).

Nevertheless, for all the EPS evaluated the principal sugars constituents continued to be neutral like glucose and mannose (Figure 3.5). Glucose is in all EPS from all strains in values between 15.43 %mol and 47.54 %mol. Mannose is the sugar in higher concentration, in a range of 4.70 %mol to 73.68 %mol. A hexose also common in marine bacteria is rhamnose, however only appears in EPS synthesized by bacteria SPUR-39, SPUR-34 and SPUR-69 with 14 %mol, 2.96 %mol and 29.5 %mol, respectively. These first two strains showed a similar EPS composition, may belong to bacteria of the same species.

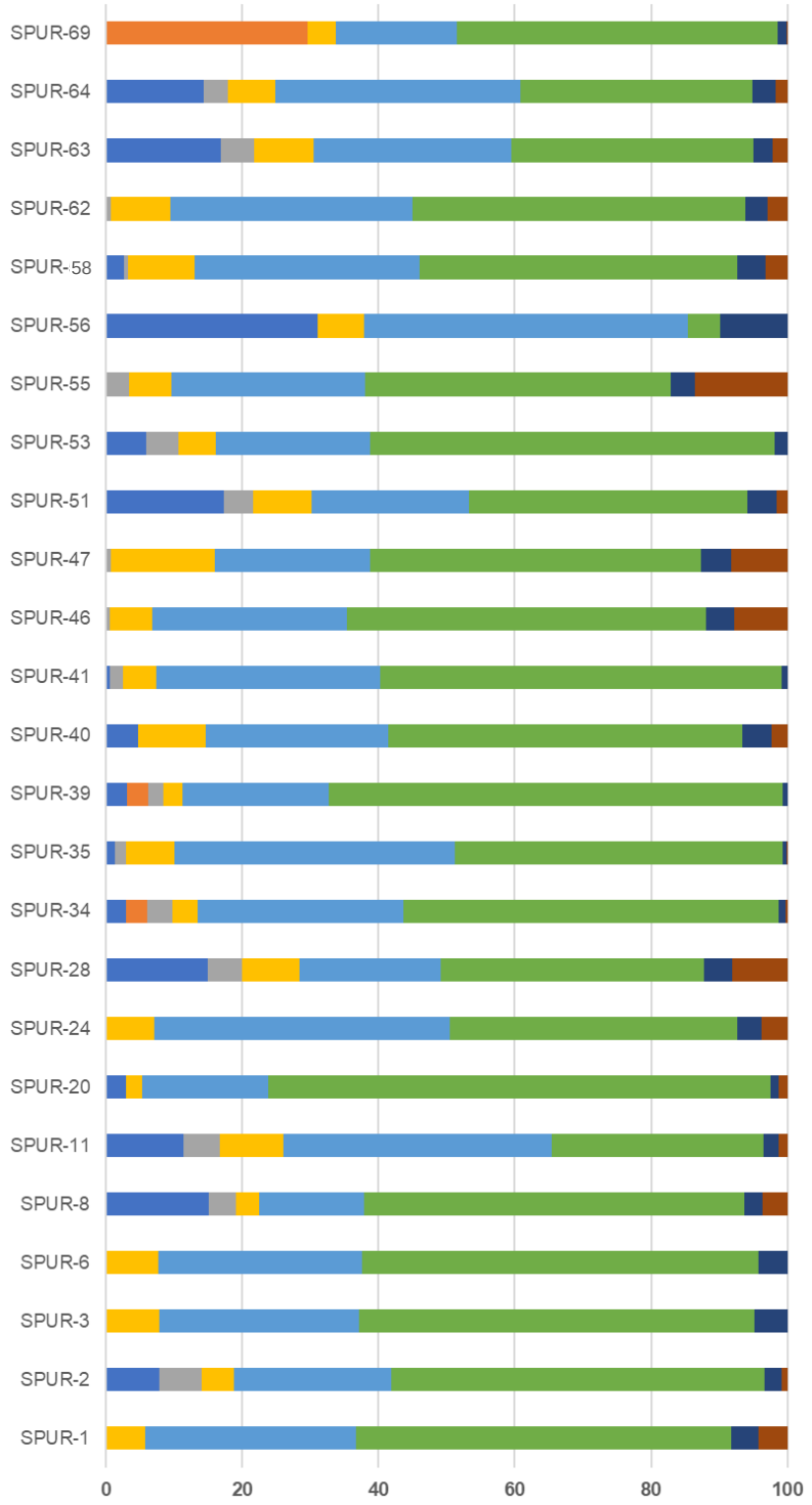

Figure 3.5 – Sugar monomer composition of EPS synthesized by SPUR in 200mL A1 SSW. (●) Fucose; (●) Rhamnose; (●) Arabinose; (●) Galactose; (●) Glucose; (●) Mannose; (●) Glucuronic Acid; (●) Galacturonic acid

Fucose was obtained in an increased number of EPS when compared with 30mL assays. Inclusive, SPUR-56 shows on its composition 31.10 %mol, which is a very interesting concentration. Fucose is a rare sugar, difficult to obtain in nature. It is known that fucose may confer to the polymer biological activity such as forming capacity, hydrophilic with high permeability to water vapour and good barrier properties to gases (CO_2 and O_2) (Ferreira et al., 2016). For instance, FucoPol is a fucose-rich EPS which exhibit interesting functional properties like forming viscous aqueous solution with shear thinning behaviour, film-forming capacity, flocculation activity, emulsion forming and stabilizing capacity (Ferreira et al., 2016; Torres et al., 2015; Freitas et al., 2014). Other important bacterial fucose-containing EPS is Fucogel, produced *by Klebsiella pneumonia* I-1507 and it is commercialized by the cosmetic industry because to their thickening, emulsifying and film-forming properties (Freitas et al., 2011).

In the majority of the EPS, arabinose was also detected in amounts between 0.63 %mol and 6.28 %mol. The uronic sugars, such as glucuronic and galacturonic acid continue to be in all strains, SPUR-55 report 17 %mol of this rare acid sugars. Uronic acid sugars are also very interesting since may impart important properties to cosmetic and medical industries. As mention above, a hyaluronic acid-like produced by a marine bacteria *Vibrio diabolicus* is commercially available because of the great capacity to restoring bone and skin and the ability to accelerate in vitro collagen fibrillation and active fibroblasts (Holmostrom, 1999).

Comparing the EPS composition of studied bacteria with literature there are many reports about EPS that are heteropolysaccharides, where mannose and glucose are the dominant sugars. However, very few marine bacteria had fucose in their composition. One example is Bramhachari et al., 2006 that report a marine bacterium, *Vibrio harveyi* strain VB23 that produced an EPS with emulsifying properties and exhibit fucose in our composition. The content in glucuronic acid is also very common in this type of bacteria (Radchenkova et al., 2013; Roca et al., 2016). Caruso et al., 2019 report a bacterium, *Marinobacter* sp. W1-16 from Antarctic surface seawater, that produced an EPS compose by glucose, mannose, galactose, galacturonic acid and glucuronic acid with emulsifying, cryoprotective and heavy metal binding properties.

3.2.1.2. PHA characterization

Despite the visualization of fresh samples stained with Nile Blue dye under optical microscope. Biomass samples of the assays in 200 ml medium A1 with SSW were also evaluated by gas chromatography.

Results demonstrated that all bacteria have HB and HV monomers in its composition, however it was not possible to determine the concentration of each monomer because during the GC analysis, problems with the hydrogen line, affected the spikes size of monomers and internal standard. The result was the most expected since HB is the most common and extensively studied monomer in PHA (Rivera-Briso et al., 2018).

Studies by Baptista (2013) showed that bacteria isolated from marine ecosystems from Madeira Archipelago are capable to produce PHA, with HB and HV monomers in its composition.

3.2.2. Assays in medium A1 with Fresh Sea Water (FSW)

In this assay the synthetic sea water was replaced by fresh sea water (FSW) and deionized water in the proportion 75:25 in order to evaluate the impact on bacterial growth and biopolymer production.

Taking into consideration the cellular growth and EPS production obtained in the previous assays, four bacteria were selected, namely SPUR-8, SPUR-41, SPUR-55 and SPUR-64. Bacteria SPUR-55 reached a higher growth than in SSW, namely 1.4 ± 0.05 g/L(Figure 3.6). However, the highest increase was obtained for bacteria SPUR-41, with a CDW of 1.87 g/L that was more than twice of the CDW achieved in SSW. These results can be consequence from the presence of some essential micronutrient to growth of bacteria that is not present in the components of SSW.

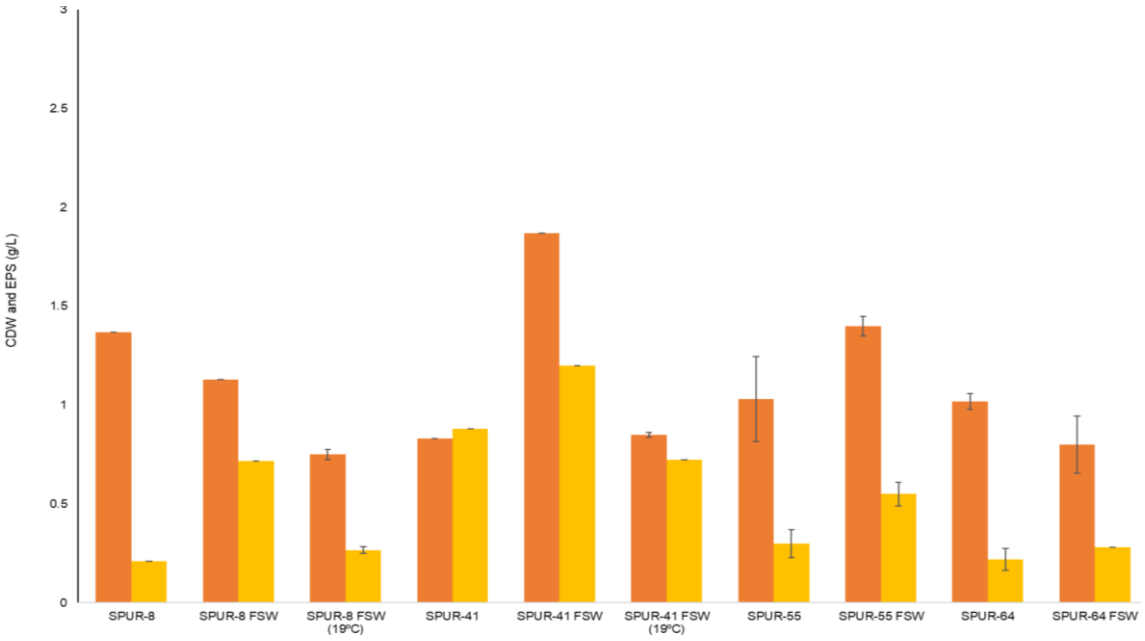

Figure 3.6. Comparation of CDW (●) and EPS (●) (g/L) produced by selected SPUR bacteria analysed in 200mL medium A1 in SSW/ FSW, at 30ºC and 19º

The exception were SPUR-8 and SPUR-64 that had attained a smaller CDW when compared with the CDW achieved using SSW.

Concerning EPS production, it was increased for all the four tested bacteria when compared with the assays in SSW. Bacteria SPUR-8, despite the lower cellular growth in FSW medium, achieved an increased EPS production. In fact, when compared with SSW, the EPS concentration achieved is more than the triple, increased from 0.21 g/L to 0.72 g/L in FSW. SPUR-41, remain to be the bacteria that synthesize a higher EPS production, with a final EPS of 1.2 g/L. The only bacteria that showed a different profile was SPUR-64, the EPS production was similar in both waters (SSW and FSW). Results, seems to demonstrate that salt concentration in FSW can be more favourable for EPS production. A study performed by Ko et al, (2000) reports the effect of NaCl, (0%-20% w/v), in the production of EPS by a marine microorganism, strain 96CJ10356, concluding that the ideal concentration for EPS production was 1% (w/v). The increase in the NaCl concentration led to a decrease in EPS production (Ko et al., 2000). In this work, a concentration of NaCl of 2.3% (w/v) in SSW was used, while in FSW the final concentration was 1.0% (w/v). These data suggest that tested SPUR bacteria also favour a salt concentration of 1%.

In order to evaluate the effect of temperature in the EPS production, experiments were performed at 19ºC for bacteria SPUR-8 and SPUR-41, the ones with the higher EPS production in FSW, at 30ºC (Figure 3.6). It was expected that incubation at lower temperatures will result in an enhancement of EPS production and reduction of cellular growth rate and cellular mass, resulting in long exponential phase and higher viscosity (Kojic et al., 1992). Sutherland proposed a mechanism to explain this phenomenon, a decrease in temperature causes a decrease in growth rate and cell wall polymer biosynthesis, resulting in more precursors available for EPS synthesis. Therefore, in the assays at 19ªC, SPUR-8 and SPUR-41showed lower cellular growth (0.08 and 0.85 g/L, respectively), with lower specific cellular growth rates (0.04 and 0.06 h^{-1}). However, the production of EPS was also lower than at 30ºC (0.27 and 0.73 g/L). This can be due to the fact that EPS produced by bacteria are associated with cellular growth, hence with the decrease in growth rates, there is also a decrease in EPS synthesis. Or the fact that despite being isolated from a cold environment, bacteria have the optimum EPS production at a higher temperature. As reported by Llamas et al. (2010) for marine bacterium where it was demonstrated that the optimum temperature for EPS production is 32ºC when compared with 22ºC.

3.3 Bioreactor cultivation

The cultivation of bacteria in bioreactors offers a multitude of advantages compared to shake flasks. The objective of shake flasks tests was to define nutritional requirements and cultivation conditions to improve EPS and PHA production. However, in shake flasks it was not possible to monitor or control essential parameters (e.g. pH, aeration, stirring). The advantages of bioreactor include the reduction of the contamination rate, stability of the strain, control of the cellular growth, and constant conditions during the process (Lillo et al., 1990).

From a total of 67 bacteria screened for EPS production and PHA accumulation, 4 were selected for cultivation in bioreactor, namely bacterium SPUR-8, SPUR-41, SPUR-55 and SPUR-64 (Figure 3.7). These bacteria are the one that showed a more promising results regarding cellular growth, biopolymer production and EPS sugar monomers composition. Therefore, in order to obtain a better knowledge about the different bacteria growth kinetic and biopolymer production batch tests were with FSW and deionized water in the proportion 75:25. Further, with the aim of increasing biopolymer production the initial glucose concentration was increased to 20 g/L, with the exception of SPUR-55 (15g/L of glucose).

The taxonomic characterization of the four SPUR bacteria was performed. The 16S rRNA gene sequencing analysis revealed 99% homology of SPUR-64 with *Bacillus licheniformis* strain DSM 13. In addition, SPUR-41 by the 16S rRNA gene sequencing analysis revealed to be *Brevundimonas* sp. and SPUR-55 a *Bacillus* sp. Bacterium SPUR-8 was not possible to characterize due to a low cellular concentration of the sample.

For all the four bacteria, the duration of the assays was different (Figure 3.7), as the criteria used to finish the runs was the continuous increasing of dissolved oxygen (data not shown). Usually, this DO increase indicates carbon exhaustion or decrease of the cellular metabolism due to loss of cellular viability. The assays took between 27 and 48 hours (Figure 3.7) and were only ceased when DO reached values above 70%.

Figure 3.7 presents the cultivation profiles of SPUR-55 (Figure 3.7 (A)), SPUR-41 (Figure 3.7 (B)), SPUR-8 (Figure 3.7 (C)), and SPUR-64 (Figure 3.7 (D)). These bacteria grew in a batch cultivation mode to improve the knowledge about its kinetic parameters and try to improve EPS and PHA production.

The strain that presented the highest growth was SPUR-55 (Figure 3.7 (A)), yielding a maximum CDW of 2.71 g/L, in approximately 30 hours after the beginning of assay and a maximum specific growth rate of 0.245 h^{-1}. This bacterium was the only one that started the cultivation with 10 g/L glucose. Thus, after 10h, when DO data start increasing (data not shown) indicating the culture could be entering in the stationary phase, a pulse with 5 g/L of glucose was added. The purpose of this strategy was to add more carbon to the medium so that the bacteria could use it to produce more EPS and PHA.

SPUR-8 (Figure 3.7 (C)) was the strain that presented the highest specific growth rate, reaching 0.579 h^{-1} with a maximum CDW of 2.09 g/L. The other strains, SPUR-64 (Figure 3.7 (D)) reached slightly lower CDW value of 1.45 g/L (maximum specific growth rate of 0.306 h^{-1}) and SPUR-41 (Figure 3.7 (B)) reached a growth of 2.29 g/L at 7 hours of run.

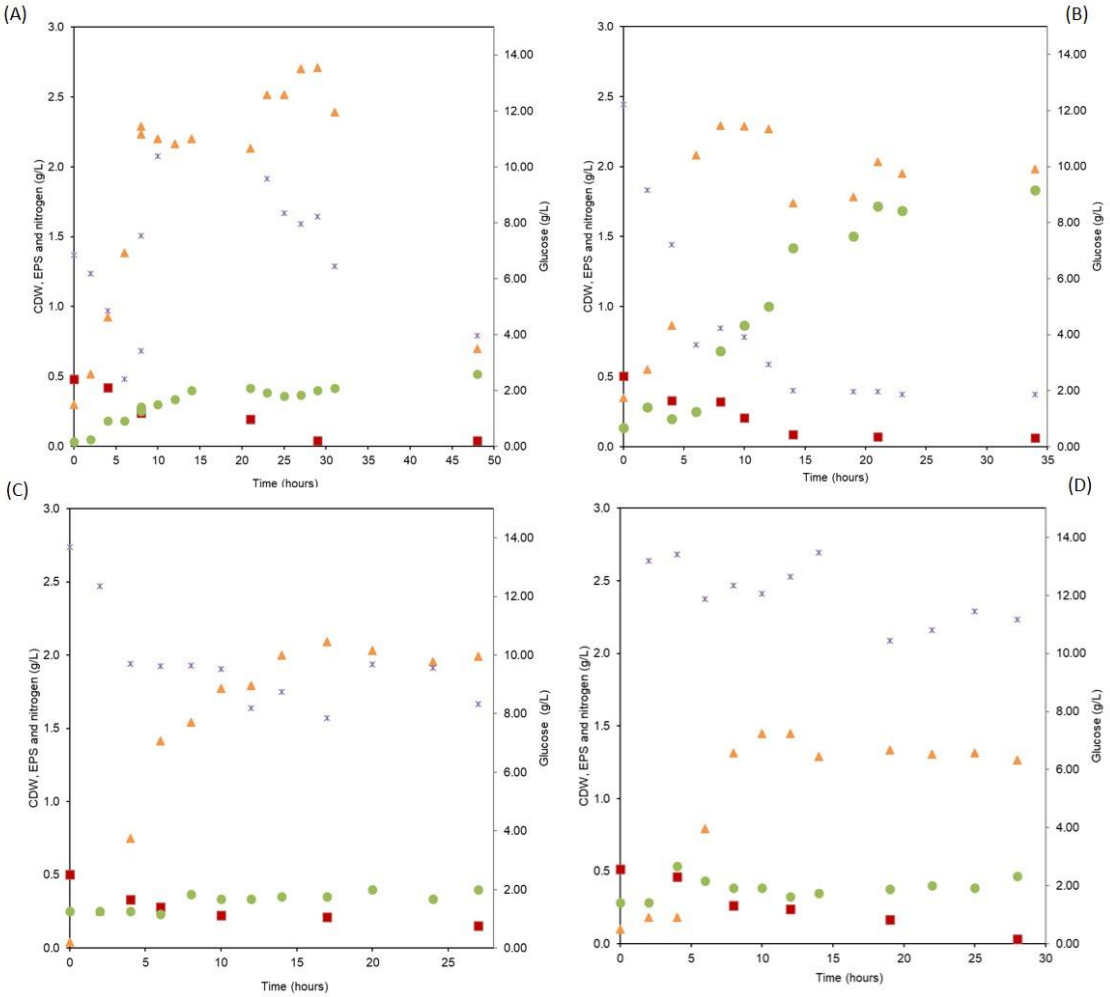

Figure 3.7 - Cultivation profile of SPUR-55 (A), SPUR-41 (B), SPUR-8 (C) and SPUR-64 (D) with A1 FSW culture medium at 30ºC. (∗) Glucose (g/L); (■) Nitrogen (g/L); (●) EPS (g/L); (▲) CDW (g/L)

In all runs, cell growth occurred in the first 7 hours of cultivation (SPUR-55, -41 and -64) and 16 hours for bacterium SPUR-8. However, glucose consumption is very reduced during that period of time. Hence, in all cases, except in SPUR-41 (Figure 3.8) (that showed a higher consume in the first hours), cells were probably using yeast extract and/or the peptone present in the medium as carbon source.

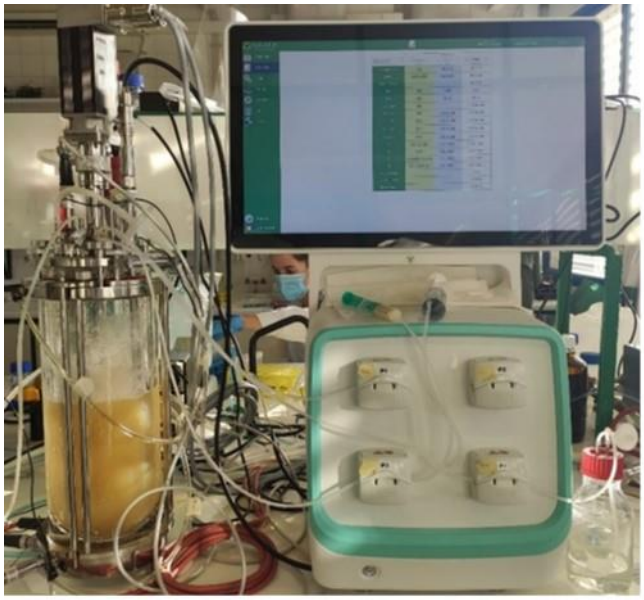

Figure 3.8 – Bioreactor with SPUR-41 culture, during FSW assay with 20g/L of glucose, at 8 h fermentation.

In terms of glucose consumption, glucose was never totally consumed. Moreover, the initial nitrogen concentration was low (0.5 g/L) then when bacteria stopped growing is when nitrogen begins to be a limiting factor. Nevertheless, all bacteria tested in bioreactor achieved higher CDW than in the 200mL shake flask cultivations. A better control of growth parameters, such as constant pH, higher glucose supplementation, higher availability of oxygen and higher homogenization of the medium that increased the mass transfer of nutrients and oxygen, contributing for an enhanced cellular growth.

Regarding EPS production, 0.48 g/L, 1.83 g/L, 0.40 g/L and 0.48 g/L of EPS were produced by SPUR-55, -41, -8 and -64, respectively. In the case of SPUR-41, EPS production started after 2 hours, however, production became more significant when nitrogen became a limiting factor, during the stationary phase. However, in all other strains, EPS was produced in the first hours of the exponential phase, when nitrogen was still in the medium, so, we can consider this bacteria growth associative. Samain et al. describe a bacterium that show this behaviour using the same nitrogen sources (peptone and yeast extract) in their assays (Samain et al., 1997). The strategy used was changing the nitrogen sources to ammonium chloride. This source was added to the assay over the time of fermentative process on the form of pulses with low concentration. In other words, the concentration of nitrogen was enough to keep growth of bacteria and EPS production. It is important to note that the low production of EPS can be due the small consumption of carbon source, since glucose seems to be a non-preferential carbon source for these bacteria.

In the end, SPUR-55 showed an EPS production of 0.48 g/L in bioreactor, lower than the one in 200 mL assay. This finding might be justified by the competition occurring during the growth phase between EPS and cell- wall polymer biosynthesis (Sutherland, 1982). In other words, this

bacterium may not produce so much EPS because has given priority to produce polymers to synthesis of cell wall and consequently for growth. Which makes sense, because was the bacteria that showed a higher CDW.

In the beginning of SPUR-8 (Figure 3.7 (C)) and SPUR-64 (Figure 3.7 (D)) assay, right after the inoculum, the EPS concentration was 0.25 g/L and 0.28 g/L, respectively. This are values quite high and not normal for the starting of assays. However, this can be justified by the presence of protein in the medium, because is a very rich medium composed with peptone and yeast extract. During the purification of EPS by dialysis, protein with bigger size than the membrane pore size probably does not step off and interfere with quantification of total EPS.

Considering the total time of the cultivation assay, a volumetric production of 0.48 g/L, 1.83 g/L, 0.40 g/L and 0.48 g/L (Table 3.1) was achieved in SPUR-55, -41, -8 and -64 respectively.

For instance, *Pseudomonas* sp. MD12-642, isolated from Artic sea ice of the Canada basin, produced 0.57 g/L of EPS in 3 days, corresponding to a productivity of 0.19 g_{EPS}/L d^{-1}. Also, a productivity of 0.032 g_{EPS}/L d^{-1} was achieved in 5 days cultivation *Halomonas elongate* when growth on a cultivation medium with glucose as carbon source (Table 3.1).

Brevundimonas dimituta, a strain with the same genus than SPUR-41, produced 0.41 g/L of EPS in 5 days, corresponding to a productivity of 0.40 g_{EPS}/L d^{-1} (Djolo et al., 2019). However, SPUR-41, in this study, showed higher productivity, 1.30 g_{EPS}/L d^{-1} .

When compared with other *Bacillus licheniformis* S14, the EPS volumetric productivity by SPUR-64, using only glucose as carbon source, was higher, 0.23 g_{EPS}/L d^{-1} and 0.41 g_{EPS}/L d^{-1}. The EPS production reported was 0.36 g/L (Spanò et al., 2013).

On the other hand, there are marine bacteria that have shown higher performances, such *Pseudomonas* sp. MD12-642, which produced 2.5 g/L of EPS within 15 hours of cultivation with glucose, corresponding to a productivity of 1.56 g_{EPS}/L d^{-1}. This can be because the quantity of glucose using in Roca et al., study was slightly higher (30 g/L), than the one used in this study (Roca et al., 2016). Furthermore, according to Grobben et al., EPS synthesis is favoured by an excess of carbon source in association with limitation of another nutrient (e.g., nitrogen) (Grobben et al., 1998). However, *Pseudomonas* sp. MD12-642 in fed-batch, yielded the highest reported production, with an EPS production of 4.4 g/L within 17 hours of cultivation on glucose, corresponding to a volumetric productivity of 6.19 g_{EPS}/L d^{-1}. (Roca et al., 2016).

In summary, EPS production was improved for strains SPUR-41 and SPUR-64 during bioreactor cultivation. In shake flask, SPUR-41 and SPUR-64 produced 0.73 g/L and 0.30 g/L, respectively. The fact that SPUR-8 and SPUR-55 did not improved the EPS production could be because pH 7 is not the best condition for these strains and the glucose was not the ideal carbon source.

SPUR-41, which was the highest EPS producer in the shake flasks cultivation, reached the highest production of 1.83 g/L during bioreactor cultivation.

Table 3.1: Marine bacteria EPS production studies comparison

Strain	Location	Carbon Source	Cultivation time (h)	CDW (g/L)	μ (h^{-1})	EPS (g/L)	r_{EPS} (g/L d^{-1})	Reference
SPUR-55 Batch	Ocean sediments SPUR Estremadura	Glucose	48	2.71	0.26	0.48	0.24	This study
SPUR-41 Batch	Ocean sediments from SPUR Estremadura	Glucose	34	2.29	0.25	1.83	1.30	This study
SPUR-8 Batch	Ocean sediments from SPUR Estremadura	Glucose	27	2.09	0.58	0.40	0.35	This study
SPUR-64 Batch	Ocean sediments from SPUR Estremadura	Glucose	28	1.45	0.31	0.48	0.41	This study
***Brevundimonas diminuta* Batch**	Marchica lagoon in Morocco	Glucose	120	0.7	n.a.	2	0.40	Dosoounon et al., 2019
***Pseudomonas* sp. MD12-642 Batch**	Ocean sediments from Madeira Archipelogo	Glucose	15	n.a	0.60	2.5	1.56	Roca et al., 2016
***Pseudomonas* sp. MD12-642 Fed-batch**	Ocean sediments from Madeira Archipelogo	Glucose	17	n.a	n.a	4.4	6.19	Roca et al., 2016
***Halomonas elongate* Batch**	Sehline Kerkennah Salt Lake	Glucose	120	n.a	n.a	0.16	0.03	Joulak et al., 2019
Pseudoalteromonas SM203010 Batch	Artic sea ice of the Canada Basin	Glucose	72	n.a	n.a	0.57	0.19	Li et al., 2013
Bacillus licheniformis S14 Batch	Shallow vent off Panarea Island	Sucrose	48	n.a	n.a	0.36	0.18	Spanò et al., 2013

n.a – Data not available

3.3.1 EPS sugar monomer composition

The EPS obtained in strain SPUR-55, -41, -8 and -64 were characterized in terms of sugar composition (Table 3.2).

Table 3.2 - Monosaccharide profile (in %mol) of the EPS produced by the isolates SPUR-8, SPUR-41, SPUR-55 and SPUR-64 during batch cultivation.

Bacteria	**Sugar Composition (%mol)**							
	Rhamnose	Arabinose	Galactose	Glucose	Mannose	Glucuronic acid	Galacturonic acid	Fucose
SPUR-8	2.74	4.73	2.19	22.08	65.83	1.07	0.16	1.18
SPUR-41	19.41	-	2.53	9.19	62.55	4.43	1.89	-
SPUR-55	4.48	-	1.93	8.59	63.72	17.21	4.06	-
SPUR-64	1.72	-	6.39	31.61	59.94	1.75	1.15	0.42

The results obtained in this study indicate that the polymer produced by the bacterium SPUR-8 was mostly composed of mannose (65.83 %mol), glucose (22.08 %mol), arabinose (4.73 %mol), rhamnose (2.74 %mol), galactose (2.19 %mol) and presented a small content in glucuronic acid (1.07 %mol), galacturonic acid (0.16 %mol) and fucose (1.18 %mol). The EPS produced by SPUR-41 was mostly composed of mannose (62.55 %mol), glucose (9.19 %mol), rhamnose (19.41 %mol), glucuronic acid (4.43 %mol) and presented a small content in galactose (2.53 %mol) and galacturonic acid (1.89 %mol). The EPS produced by SPUR-55 was mostly composed of Mannose (63.72 %mol), glucuronic acid (17.21 %mol), Glucose (8.59 %mol), rhamnose (4.48 %mol) and presented a small content in galactose (1.93 %mol) and galacturonic acid (4.06 %mol). Finally, SPUR-64 was composed by mannose (59.94 %mol), glucose (31.61 %mol), galactose (6.39 %mol) and presented a small content in glucuronic acid (1.75 %mol), galacturonic acid (1.15 %mol), rhamnose (1.72 %mol) and fucose (0.42 %mol).

Nevertheless, Le Costaouec et al., 2012, reported a marine bacterium, *Alteromonas macleodii subsp. Fijesis* biovar deepsane HYD 657, isolated from deep sea hydrothermal vent that produce an EPS with a composition of mannose, fucose, rhamnose, glucose, galacturonic acid, galactose and glucuronic acid, very similar with SPUR-64 monosaccharides composition.

In the literature, marine bacteria that produce uronic acids have already been reported (Le Costaouec et al., 2012; Roca et al., 2016; Zhang et al., 2015). As mention above, the presence of uronic acids in such high content, especially glucuronic acid, may render the EPS interesting properties for biotechnological uses with important properties to cosmetic and medical industries. *Brevundimonas dimituta*, a strain with the same genus than SPUR-41, was reported as a producer

of EPS that had shown 20% antiproliferative against myeloid cancer and flocculating activity of 66% (Djolo et al., 2019).

Furthermore, polysaccharides possess non sugar substituents, namely organic acyl groups (e.g. acetyl, succinyl and pyruvyl), components frequently found in microbial EPS, confers the polymers polyelectrolyte character (Freitas et al., 2011). Additionally, they greatly influence polymer's properties, namely, solubility and rheology (Rinaudo, 2004). Analysis to these groups was performed, however it was only possible to assure the presence of acetyl, succinyl and pyruvyl, but not their quantity.

3.3.2 PHA Composition

Regarding PHA production, it was evaluated by optical microscope observations as in shake flask assays. In figure 3.9, it is possible to observe the PHA granules accumulated inside the bacterial cells of the bacteria SPUR-55 (Figure 3.9 (A)), -41 (Figure 3.9 (B)), -8 (Figure 3.9 (C)), -64 (Figure 3.9 (D).

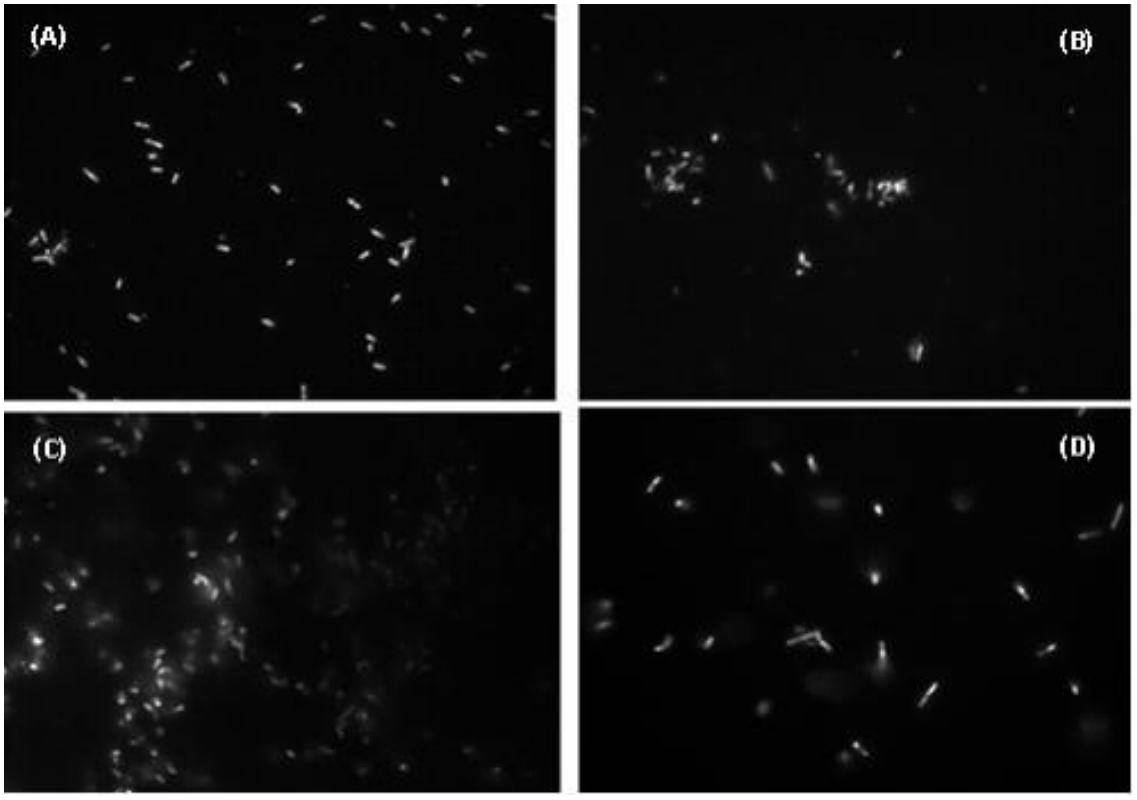

Figure 3.9 - Fresh samples of bacteria with Nile Blue solution at the end of exponential phase (100x); (A) SPUR-55; (B) SPUR-41; (C) SPUR-8; (D) SPUR-64

During the GC analysis a problem with hydrogen line occurred and affected the spikes size of monomers and internal standard, therefore it was only possible to quantify the monomers.

Results demonstrated that all bacteria have HB and HV monomers in its composition. This could be related with the presence of co-polymer poly(3hydroxybutyrate-co-hydroxyvalerate) (P(3HB-co-3HV)) that, as discussed above, have great potential in the medical and pharmaceutical fields. Co-polymers have a higher degradation rate, a rougher surface and low crystallinity compared to homopolymers. The surface morphology of co-polymers combined with the advantage of low crystallinity and rough surface enables it to degrade faster in the environment, so this polymer is also used in a variety of uses as single uses like bulk-commodity plastics (Nanthini Sridewi et al., 2006).

Marine bacteria *Halomonas mediterranei* DSM 1411, is able to produce (P(3HB-co-3HV)) with various carbon source like vinasse, rice-based ethanol and hydrolyzed cheese whey (Pais et al., 2015; Bhattacharyya et al., 2012; Bhattacharyya et al., 2014).

The monomeric composition of PHA from SPUR-41, a bacterium from *Brevundimonas* sp., contained HB and HV. These results were similar to those reported by Silva et al., 2003 with *Brevundimonas vesicularis* LMG P-23615. In relation to SPUR-64, *Bacillus licheniformis* strain DSM 13, it was not found any reports about PHA production. In addition, it was found that a halophilic *Bacillus* sp. use diverse carbon sources in order to produce PHA such as P(3HB) and P(3HB-co-3HV) (Moorkoth et al., 2016).

3.4 Conclusion and Future Work

Cellular growth EPS and PHA production by 67 bacteria isolated from Estremadura SPUR marine sediments were evaluated.

In shake flask assays all the 67 strains studied showed ability to co-produce EPS and PHA. Therefore, the 25 strains that exhibited the most promising results in the 30 mL assays were tested in 200 mL of medium A1 prepared with synthetic sea water and with fresh sea water. The best results for cellular growth and biopolymer production were achieved for using FSW at 30ºC rather than SSW and 19ºC. The cultures were able to produce 0.30 – 1.2 g/L of EPS with different sugar compositions, most of them were heteropolysaccharides composed of neutral and acidic sugars. About PHA accumulation bacteria were able to accumulate a co-polymer of HB-HV.

The 4 strains that present the best results were SPUR-55, SPUR-41, SPUR-8 and SPUR-64. These bacteria studied in bioreactor under controlled conditions of pH (7), temperature (30ºC), aeration (1vvm) and pO2 (20%). Analysis of 16S rRNA sequencing revealed that SPUR-55 belongs to *Bacillus* sp. genus, SPUR-41 *Brevundimonas* sp. and SPUR-64 is a *Bacillus licheniformis* strain DSM 13.

In the bioreactor experiments, the selected cultures attained cellular growth between 1.45 and 2.71 g/L. Further, bacteria were able to produce 0.40 and 1.83 g/L of EPS corresponding to volumetric productivities of 0.35-1.30 (g/L d^{-1}). The highest EPS production was achieved for strain SPUR-41 (1.83 g/L) in batch bioreactor and P(HB-co-HV) accumulation. The EPS synthesized by SPUR-41 were composed of monomers of mannose (62.55 %mol), glucose (9.19 %mol), rhamnose (19.41 %mol), glucuronic acid (4.43 %mol) and a small content in galactose (2.53 %mol) and galacturonic acid (1.89 %mol). Moreover, it has also acyl groups in its composition, namely acetyl, succinyl and pyruvyl.

The EPS produced by bacterium SPUR-8 was composed of mannose (65.83 %mol), glucose (22.08 %mol), arabinose (4.73 %mol), rhamnose (2.74 %mol), galactose (2.19 %mol) and presented a small content in glucuronic acid (1.07 %mol), galacturonic acid (0.16 %mol) and fucose (1.18 %mol). Bacterium SPUR-55 produced an EPS with mannose (63.72 %mol), glucuronic acid (17.21 %mol), glucose (8.59 %mol), rhamnose (4.48 %mol), galacturonic acid (4.06 %mol) and presented a small content in galactose (1.93 %mol) and bacterium SPUR-64 synthesize an EPS composed of mannose (59.94 %mol), glucose (31.61 %mol), galactose (6.39 %mol) and presented a small content in glucuronic acid (1.75 %mol), galacturonic acid (1.15 %mol), rhamnose (1.72 %mol) and fucose (0.42 %mol).

Some of the achieved results are very promising however some work on the optimization of the fermentation conditions is needed to improve PHA and EPS production. It was clear from the obtained results that glucose was not fully consumed by microorganisms, which may have directly compromised the production of EPS and PHA. According to the literature, marine bacteria may prefer other substrates than glucose (e.g lactose, maltose, sucrose, starch, galactose). Test other cultivation media, such as LB, Shatz, etc. The salt concentration should also be optimized, as well as nitrogen source and concentration. Further, the optimal temperature and pH should

also be studied.

It will be important to evaluate the impact of different bioreactor operation modes and feeding strategies, namely fed-batch or continuous mode with pulse or continuous feeding supplementation.

Further work is necessary to better characterize EPS, such as in terms of molecular weight and total protein content. Analyse the PHA molecular weight is also important.

Chapter 4
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